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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

G-protein coupled receptors (GPCRs) receive many of the neural, hormonal, and paracrine inputs to activate different types of cells, regulating intracellular second messengers and a variety of cellular functions, including cell growth and proliferation. Constitutive activation of a number of G-protein coupled receptors have been linked to the proliferative signaling pathways and a number of human neoplasias. Most recently, we have identified a novel prostate-specific G-protein coupled receptor (PSGR). Northern blot analysis indicates that the expression of PSGR is increased significantly in human prostate cancers compared to normal prostate tissues, suggesting a potential role of PSGR in prostate tumor progression and development. We have generated mutant receptors for our further studies of the PSGR signaling pathway. We have also characterized the dual promoter control of human PSGR gene in our in vitro assays. Furthermore, we have generated a transgenic construct for our future studies on the role of PSGR in prostate cancer in mouse model. The results obtained will help us understand the mechanism of prostate cancer prevention and therapy.

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INTRODUCTION

Receptors coupled to heterotrimeric GTP-binding proteins (G-proteins) are integral membrane proteins involved in the transmission of signals from the extracellular environment to the response machinery in the cell cytoplasm and nucleus. A variety of external stimuli, including n eutrotransmitter, h ormones, p hospholipids, p hotons, o dorants, t aste ligands, and growth factors, can activate the G-protein coupled receptors (GPCRs). Any disruption in the normal signaling processes of the cells will lead to the disease conditions, including prostate cancers. Therefore, the G-protein coupled receptors and signaling machinery represent important specific targets for a variety of therapeutic approaches, ranging from the control of blood pressure, allergic response, kidney function, and hormonal disorders, to neurological diseases and chronic pain. It has been estimated that about 40% of the pharmaceutical products sold in the United States target the G-protein mediated signal transduction systems. Prostate cancer is now the most common cancer among men in the US. Cancer cells escape the normal growth controls by disordered intracellular signaling pathways that allows these malignant cells to proliferate and migrate. Therefore, drugs that target to the specific signaling machinery only in the prostate cancer cells might have significant therapeutic benefits against this form of cancer. Most recently, we have identified a novel prostate-specific G-protein coupled receptor, called PSGR. This receptor protein is only existed in human prostate tissue, not in other organs. Furthermore, in prostate tumors, this specific protein is dramatically increased compared to normal prostate tissues, suggesting that this unique prostate receptor protein might participate in the proliferation and progression of prostate cancers.

BODY

Objective/Hypothesis: The overall hypothesis is to test the idea that the prostate-specific receptor PSGR and its specific signaling pathway(s) are essential for the maintenance of prostate cell growth and proliferation. Overexpression or over-activation of the PSGR receptor and signaling pathways will lead to the prostate tumor development.

Task 1. Determine the role(s) of PSGR in prostate cancer cell signaling, proliferation, and tumor progression:

Generation of constitutive active PSGR receptors by site-directed mutagenesis

The constitutive activation of G-protein-coupled receptors is a major new approach to investigating their physiopathology and pharmacology. Previous studies have demonstrated that a number of residues are critical for receptor activation and are highly conserved among different G-protein coupled receptors. For example, the highly conserved DRY motif at the intracellular end of transmembrane III (TMIII) is essential for the receptor activation through protonation events of its side chain (Scheer *et al.*, 1997). Charge-neutralizing mutations at the aspartic acid lead to the generation of agonist-independent (constitutive) activation of receptors in a number of G-protein coupled receptors (Fig.1). The Kaposi's sarcoma herpesvirus-G protein-coupled receptor (KSHV-GPCR), which is known to be constitutively activated and able to cause oncogenic transformation, also has mutation at this aspartic acid residue of the DRY motif (Arvanitakis *et al.*, 1997). Therefore, mutation at this aspartic acid residue is most likely to promote the conversion of the PSGR receptor into its agonist-independent active form.

To generate constitutively active PSGR mutant receptors, we have changed the aspartate residue of the DRY motif into six different amino acids (D120A, D120M, D120N, D120K, D120T, D120I, and D120V) by site-direct mutagenesis (Fig. 1). The agonist independent activity of the mutant receptors will be tested for their ability to induce constitutive inositol phosphate (IP) accumulation in a stable-transfected COS-7 cell lines using a well-developed reconstitution assay coupled to the promiscuous $G\alpha_{15/16}$ protein (Offermanns and Simon, 1995; Liu and Simon, 1996, Xia *et al.* 2001b).

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MSSCNFTHATFVLIGIPGLEKAHFWVGFPLLSMYVVAMFGNCIV 44
                                    TMI
VFIVRTERSLHAPMYLFLCMLAAIDLALSTSTMPKILALFWFDS 88
                  TMII
REISFEACLTQMFFIHALSAIESTILLAMAFDRYVAICHPLRHA 132
                  TMIII
AVLNNTVTAQIGIVAVVRGSLFFFPLPLLIKRLAFCHSNVLSHS 176
                  TMIV
YCVHQDVMKLAYADTLPNVVYGLTAILLVMGVDVMFISLSYFLI 220
                                    TMV
IRTVLQLPSKSERAKAFGTCVSHIGVVLAFYVPLIGLSVVHRFG 264
                                    TMVI
NSLHPIVRVVMGDIYLLLPPVINPIIYGAKTKQIRTRVLAMFKI 308
                  TMVII
SCDKDLQAVGGK                                  320
```

Fig. 1. Amino acid sequence of PSGR with seven putative transmembrane domains (TMI-TMVII) of the protein. The conserved DRY are red colored.

Generation of stable cell line that expressing $G\alpha_{16}$ for receptor activation assay

It has been shown that a wide variety of receptors, including receptors coupled to Gαq family, receptors activate to Gαs and Gαi, can activate $G\alpha_{15/16}$ proteins to stimulate the production of IP_3 and intracellular Ca^{2+} (Offermanns and Simon, 1995; Gu *et al.*, 2003). Therefore, $G\alpha_{15/16}$ proteins can bypass the selectivity and specificity of receptor-G-protein interactions to activate phospholipase C (PLC). This promiscuous property of the two G-proteins has been used to examine active receptors and the potential agonists that activate the receptors. To facilitate our experiments with the constitutive PSGR receptor assays, we have transfected $G\alpha_{16}$ into the COS-7 cells and generated a stable cell line that overexpressing the $G\alpha_{16}$ protein. Once we generate the PSGR mutant receptors, we will reconstitute the receptors, $G\alpha_{15}$ or $G\alpha_{16}$ signaling pathway in COS-7 cells. The production of [^{3}H]labeled inositol phosphates (IP_3) will be determined as previously described (Offermanns and Simon, 1995; Liu and Simon, 1996). Briefly, cells will be cotransfected with PSGR receptor (wild type or active mutant receptor) and $G\alpha_{16}$ (or $G\alpha_{15}$) using lipofectamine (Life Technologies, Inc.). Approximately 24 hrs after transfection, cells will be labeled for 20-24 hrs with 120 pmol of *myo*-[2-^{3}H]inositol (Du Pont NEN) in inositol-free medium. Labeled cells will be washed with phosphate buffered saline (PBS) and incubated for 10 min with 10 mLiCl in inositol-free DMEM. Inositol phosphates released from the cells will be separated, and the production of IP_3 will be measured. The amount of IP_3 produced by wild type PSGR and the mutant receptors will be compared. The constitutive activity of the mutant PSGR receptors will be measured and the fold of increase over the control levels will be determined. Since certain mutations at this site may affect the expression level of the receptor, we will examine the expression level of different PSGR mutants using Western blot analysis with specific anti-PSGR antibody or anti-Flag M2 antibody. Using this approach, we expect to generate constitutive activation mutants of PSGR.

Task 2. Generate and characterize transgenic mouse models overexpressing PSGR

a. Characterize the promoter regions of *PSGR* gene

To understand the regulation of PSGR expression in human, we have searched the human PSGR genomic structure. Human PSGR is localized on chromosome 11p15 and is about 18 Kb long, with two exons at 200 bp and 2.6 Kb long, respectively. The two exons are separated by ~15 Kb intron (Fig. 2). Consensus TATA box is predicated in front of exon 1. To understand the regulation of PSGR expression, we characterize the potential promoter

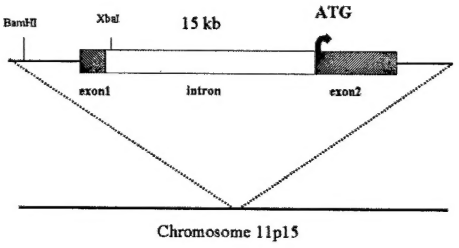

Fig. 2. Genomic organization of PSGR gene. Exon1 and exon2 are separated by a 15 kb intron.

region of PSGR by subcloning various regions in front of exon1 and exon2 into pGL3 basic vector for luciferase assays (Fig. 3).

The promoter activities of different reporter constructs were examined in different cell lines, including COS-7, 293T, HepG2, LNCaP, and PC3 cells. As shown in Fig. 4, the basic promoter is localized in front of exon1 at posititon -483 fragment (Fig. 4). An enhancer is found in between position –940 to –1.5 Kb. Also, exon1 itself has positive regulatory activity toward the basic promoter. To our surprise, we found a second promoter that localized in front of exon 2 in the long intron of exon1 and exon2. As shown in figure 3 and figure 4, the fragment to –677 of exon2 has strong promoter activity. Further characterization has shown that a small 300 bp of fragment close to exon2 has the promoter activity while a region in the long intron has silencer activity in non human prostate cells. The results obtained from the detailed characterization of the two promoters and its regulatory region of human prostate-specific G-protein coupled receptor in preparation and will be submitted for publication in 2003.

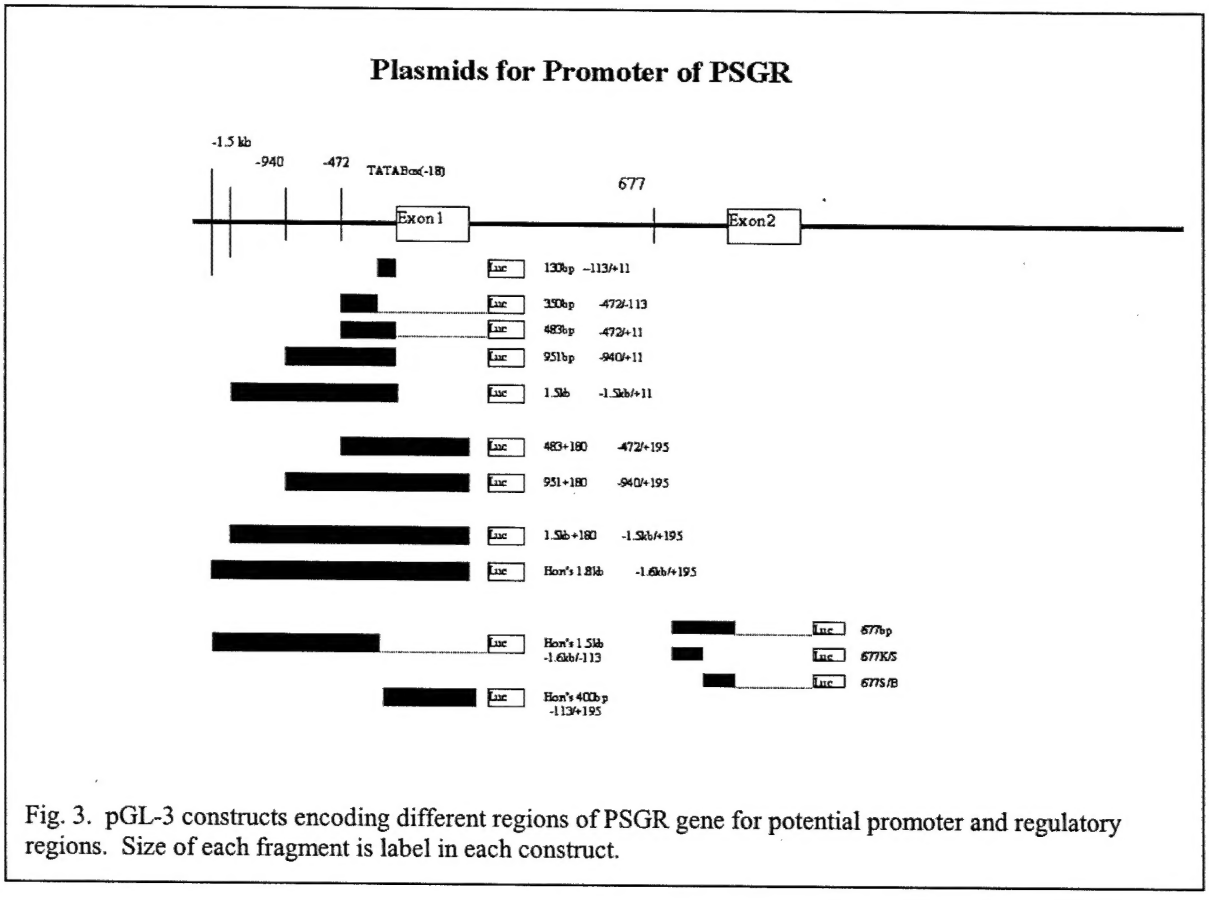

Fig. 3. pGL-3 constructs encoding different regions of PSGR gene for potential promoter and regulatory regions. Size of each fragment is label in each construct.

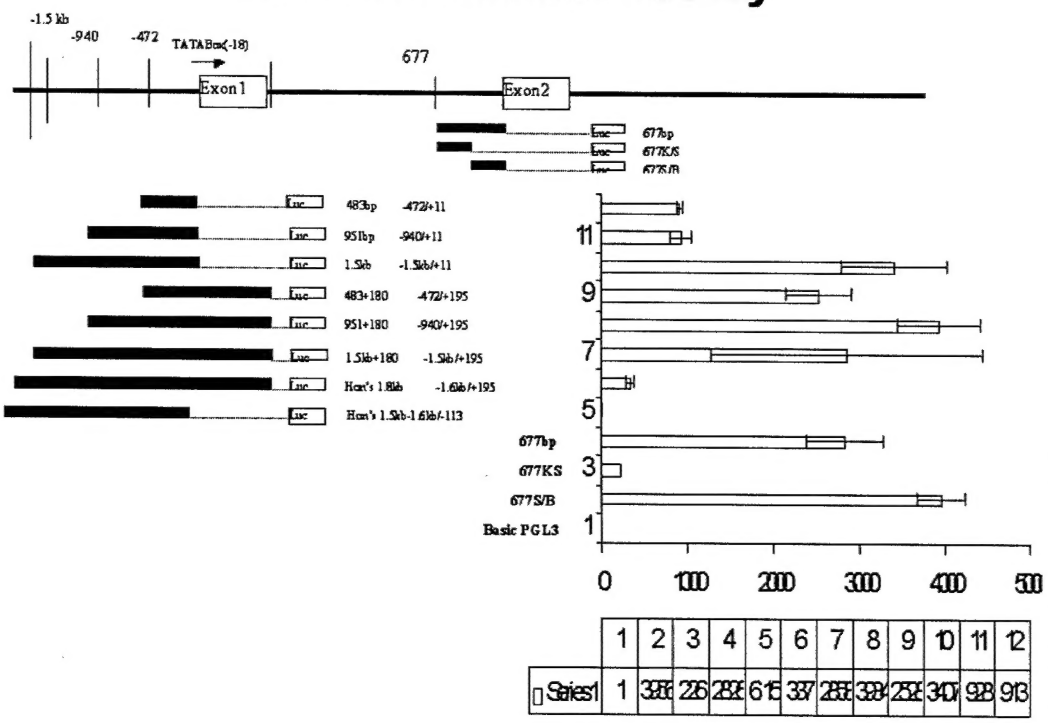

Fig. 4. Luciferase assays for different promoter and regulatory constructs of PSGR.

b. Construction of transgene constructs for PSGR

Using the modification of the pSK-PTB/SV40 vector, we have constructed the transgene vector of PSGR. In the transgenic construct, shown in figure , a well-characterized mouse probasin promoter (-426/+28) is used to drive the expression of the wild-type PSGR and its constitutive activation mutant genes. An mRNA splice site and a polyA addition site of SV40 T antigens are used to ensure the expression of the minigene. In addition, a FLAG epitope will be added in frame at the 3'-end of PSGR coding sequence to facilitate the identification of transgenic product from the endogenous PSGR (Wang, 2000). A chicken insulator element in the 5' region of the chicken β-globin locus, which associates with DNase 1 hypersensitive sites and tends to separate chromatin domains with different degrees of condensation, will be inserted at the 3'-end of poly-A addition site to ensure the expression of transgenes regardless of its insertionlocation (Fig.). It has been demonstrated that the expression of ectopic proteins in similar minigene is restricted to the prostate and is androgen dependent, which reaches the peak after the mice are sexually mature (Greenberg *et al.*, 1995). The pSK-PTB/SV40 vector has been successfully used for overexpression of several ectopic proteins in mouse prostate epithelial cells, including T-antigens in Greensburg's lab and FGFR1 in McKeehan's laboratory (Foster *et al.*, 1997; Wang *et al*, personal communication).

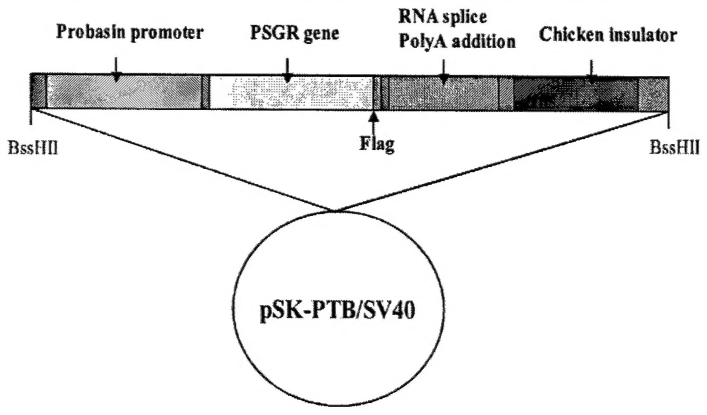

Fig. . The PSGR transgene constructure. The wild type and constitutive-active PSGR cDNAs were subcloned into the pSK-PTB/SV40 vector. The vector was digested with BssH II to release the PSGR transgene construct for prenuclear microinjection.

KEY RESEARCH ACCOMPLISHMENTS

1. We have generated a number of PSGR receptor mutants for our future functional assays.
2. We have generated cell lines that overexpress $G\alpha_{16}$ G-protein for our reconstitution assay of PSGR activation in COS-7 cells.
3. We found that there two promoters that regulate the expression of human PSGR gene and are in the process to study the regulation of the tissue specific expression of the PSGR gene.
4. We have made transgene constructs and in the process of generating transgenic mouse model that overexpress PSGR in the prostate.

REPORABLE OUTCOMES

Manuscripts/abstracts/presentations:

1) Xia, C. Ma, W., Wang, F., Hua, S-B., and **Liu, M.** 2001. Identification of a novel prostate-specific G-protein coupled receptor in prostate cancer. ***Oncogene*** **20**, 5903-5907.

2) Guo, X., Stafford, L.J., Bryan, B., Xia, C., Ma, W., Wu, X., Liu, D., Songyang, Z., and **Liu, M.** 2003. A Rac/Cdc42 specific exchange factor, GEFT, mediates cell migration, proliferation and transformation. ***Journal of Biological Chemistry*** (in press), published online on January 23rd, 2003.

3) Weng, J., Xia, C., Stafford, L., and Liu, M. 2003. Two distinct promoters drive transcription of human prostate-specific G-protein coupled receptor gene. (in preparation)

Development of cell lines:

Derivatives of LNCaP that overexpress PSGR and mutants
COS-7 cells that stably overexpress $G\alpha_{16}$

Animal models:

Transgenic mouse model are in progress.

Biomedical Sciences, obtained extensive training in gene transcription, biochemistry, cell and molecular biology, and prostate cancer research.

CONCLUSIONS:

A new prostate-specific G-protein coupled receptor designated PSGR was identified in our laboratory. The expression of PSGR is significantly increased in our tissue array and QPCR studies, suggesting a potential role of this receptor in prostate tumor development. Understanding the signaling pathway and functional role of PSGR would suggest new approaches and target in prostate cancer prevention and therapy.

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Identification of a prostate-specific G-protein coupled receptor in prostate cancer

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Membrane receptors coupled to heterotrimeric G-proteins play an essential role in the transmission of signals from the extracellular environment to the cytoplasm of the cell. A wide variety of external stimuli, including neurotransmitters, hormones, phospholipids, photons, odorants, taste ligands, and growth factors, can activate specific members of the G-protein coupled receptors (GPCRs). Besides essential functions in fully differentiated cells and tissues, GPCRs are also involved in embryogenesis, tissue regeneration, cell growth stimulation, and cell proliferation. In this study, we identified a novel prostate-specific G-protein coupled receptor that interacts with $G\alpha_{12}$ in our yeast two-hybrid assays. The expression of the receptor protein is highly restricted to human prostate tissues using multiple-tissue Northern blot analysis, and tissue expression array. Furthermore, the expression of prostate-specific receptor is increased significantly in prostate tumors in comparison with the matched normal prostate tissues using PCR and Southern blot analysis, suggesting a potential role of this tissue-specific G-protein coupled receptor in prostate cancer development. *Oncogene* (2001) **20,** 5903–5907.

Keywords: G-protein coupled receptor; GPCRs; prostate cancer; Galpha12; PSGR

Receptors coupled to heterotrimeric GTP-binding proteins (G-proteins) are integral membrane proteins involved in the transmission of signals from the extracellular environment to the cytoplasm. A variety of external stimuli, including neurotransmitter, hormones, and phospholipids, can activate the G-protein coupled receptors (GPCRs). Therefore, the G-protein coupled receptors and signal transduction pathways represent important specific targets for a variety of therapeutic approaches, ranging from the control of blood pressure, allergic response, kidney function and hormonal disorders, to neurological diseases and chronic pain (Edwards *et al.*, 2000). GPCRs share the common structural feature of a single polypeptide with seven membrane-spanning domains (Wess, 1997). Binding of specific agonists (ligands) to the receptors leads to the subsequent activation of heterotrimeric G-proteins (binding of GTP) and G-protein coupled signaling pathways. The heterotrimeric G-proteins are composed of α, β and γ subunits. The $G\alpha$ proteins comprise four subfamilies, the G_s, $G_{i/o}$, G_q, and $G_{12/13}$. Each of these groups functions to activate different subsets of effectors: the G_s and the $G_{i/o}$ regulate intracellular cAMP concentration by activating and inhibiting adenylyl cyclases, respectively; and the G_q subfamily mediates the function of phospholipase C-β (Simon *et al.*, 1991), a family of key enzymes essential for the regulation and generation of intracellular second messengers (PIP_2, IP_3, and Ca^{2+}) (Rhee and Choi, 1992).

The $G\alpha_{12/13}$ subfamily has been implicated in a number of cellular functions, including Rho-dependent cytoskeletal shape changes, activation of c-Jun N-terminal kinase, and stimulation of Na^+/H^+ exchange (Dhanasekaran and Dermott, 1996; Lin *et al.*, 1996; Collins *et al.*, 1997; Voyno-Yasenetskaya *et al.*, 1996; Gohla *et al.*, 1999). In addition, there is substantial evidence that $G\alpha_{12/13}$ proteins mediate signaling pathways involved in cell growth and tumorigenesis (Chan *et al.*, 1993; Jiang *et al.*, 1993; Jones and Gutkind, 1998; Dermott *et al.*, 1999). Whereas $G\alpha_{12}$ proteins have been directly linked to regulatory molecules such as the Rho-directed guanine nucleotide exchange factor p115 (Kozasa *et al.*, 1998; Hart *et al.*, 1998) and the GTPase-activating protein RasGAP1 (Jiang *et al.*, 1998), the significance of these interactions in relation to the developmental and oncogenic roles of $G\alpha_{12}$ proteins has not been clearly defined.

In this study, we identified a putative G-protein coupled receptor that interacts with $G\alpha_{12}$ in yeast two-hybrid assays. Northern blot analysis indicates that the expression of the receptor is highly restricted to the prostate tissues. Little or no expression was detected in other tissues. Furthermore, the prostate-specific G-protein receptor was greatly elevated in human prostate cancers compared to normal prostate tissues. Together, these results suggest that the prostate-specific G-protein coupled receptor (PSGR) may couple to $G\alpha_{12}$-mediated signaling pathway and participate in the regulation of prostate cell growth and proliferation.

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To understand the potential function of $G\alpha_{12}$ protein in prostate cancer cells, we screened a human prostate MatchMaker cDNA library using $G\alpha_{12}$ as bait. A number of positive colonies have been obtained in the yeast two-hybrid screening. One of the clones identified encodes a putative G-protein coupled receptor. Since the clone did not contain the complete protein sequence, the 5′ end of the protein was isolated by 5′ RACE–PCR. The compiled full-length cDNA contains an open reading frame of 320 amino acids with seven putative transmembrane domains (TMI to TMVII) (Figure 1). Chromosomal mapping assigned PSGR to chromosome 11p15, one of the regions that are clustered with G-protein coupled receptors. Sequence analysis indicates that the new PSGR protein shares about 30% sequence identity with various G-protein coupled odorant receptor proteins and a gustatory-specific G protein-coupled receptor (Abe *et al*., 1993).

To confirm the interaction of $G\alpha_{12}$ and the receptor, yeast cells were cotransformed with $G\alpha_{12}$ and a large fragment of PSGR receptor (amino acids 120–320) in the DNA-binding domain and the activation domain vectors, respectively. The transformed yeast cells were cultured on the selection plate (-Trp/-Leu/-His) to examine the interaction of the two proteins. As shown in Figure 2, only cells co-transformed with $G\alpha_{12}$ and the PSGR receptor can grow on the selection plates (Figure 2a,b), indicating the potential interaction of the two proteins in yeast cells. To further confirm the

A.

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MSSCNFTHATFVLIGIPGLEKAHFWVGFPLLSMYVVAMFGNCIV 44
                                  TMI
VFIVRTERSLHAPMYLFLCMLAAIDLALSTSTMPKILALFWFDS 88
                  TMII
REISFEACLTQMFFIHALSAIESTILLAMAFDRYVAICHPLRHA 132
                  TMIII
AVLNNTVTAQIGIVAVVRGSLFFFPLPLLIKRLAFCHSNVLSHS 176
                  TMIV
YCVHQDVMKLAYADTLPNVVYGLTAILLVMGVDVMFISLSYFLI 220
                                  TMV
IRTVLQLPSKSERAKAFGTCVSHIGVVLAFYVPLIGLSVVHRFG 264
                                  TMVI
NSLHPIVRVVMGDIYLLLPPVINPIIYGAKTKQIRTRVLAMFKI 308
                  TMVII
SCDKDLQAVGGK                                  320
```

B.

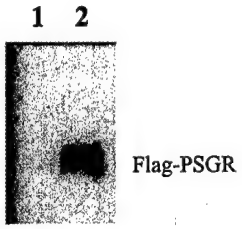

Figure 1 Amino acid sequence of PSGR and the expression of the protein in Cos-7 cells. (**a**) PSGR contains seven putative transmembrane domains (TMI-TMVII) and a conserved DRY motif in the second intracellular domain. The receptor shares approximately 50% sequence similarity with the family of olfactory receptor-like proteins in the database. The GenBank accession number is AF369708. (**b**) The Flag-tagged PSGR protein is expressed as a ~40 kDa protein on SDS–PAGE (lane 2). Lane 1 is the control cell lysate without PSGR transfection. Cos-7 cells were transfected with Flag-tagged PSGR. The cells were lysated and proteins were separated by SDS–PAGE. The expression of the PSGR protein in Cos-7 cells was detected by Western blot using M2 monoclonal antibody against Flag-epitope (Sigma)

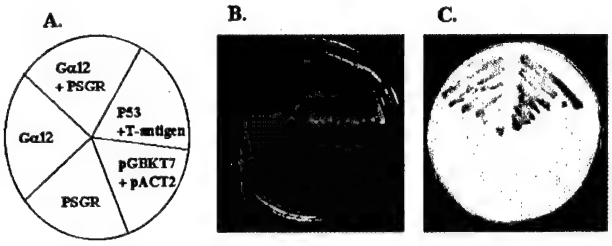

Figure 2 Interaction of PSGR with $G\alpha_{12}$ in yeast two-hybrid assays. (**a**) cDNAs encoding proteins as indicated were transformed into the yeast AH109. An activation domain plasmid pACT2 encoding the C-terminal region of PSGR (amino acids 120–320) was used in the two-hybrid assay. (**b**) The yeast cells were grown on selective synthetic medium plates (SD/-Trp/-Leu/-His) for detecting protein–protein interaction. Only cells transformed with $G\alpha_{12}$ and PSGR can grow on the selective plates. Genes encoding p53 and large T-antigen were used as positive control of the experiments. (**c**) Activation of *LacZ* reporter gene in yeast cells transformed with $G\alpha_{12}$ and PSGR in the β-gal activity assay. Yeast two hybrid system was initially used to clone PSGR. $G\alpha_{12}$ was subcloned into the Gal4 DNA-binding domain in the vector, pGBKT7. A human prostate MatchMaker cDNA library in pACT2 (Clontech Laboratories, Inc., CA, USA) was screened. Interacting proteins were selected on synthetic plates lacking four different nutrients (SD/-Trp/-Leu/-His/-Ade). The interaction was further tested by the activation of a third reporter gene, lacZ, by β-gal filter assays

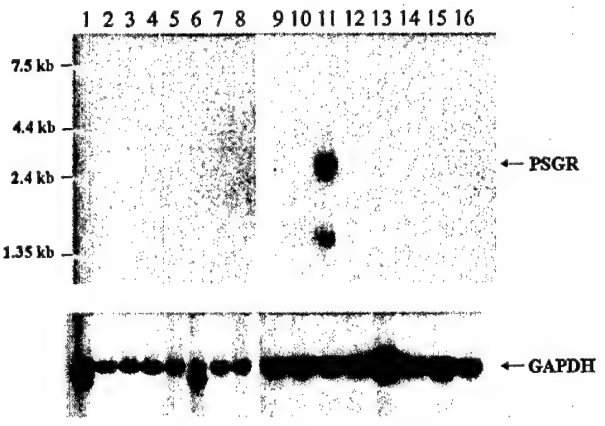

Figure 3 PSGR is expressed exclusively in the prostate tissue, not in other tissues by Northern blot analysis. (1) Heart, (2) Brain, (3) Placenta, (4) Lung, (5) Liver, (6) Skeletal muscle, (7) Kidney, (8) Pancreas, (9) Spleen, (10) Thymus, (11) Prostate, (12) Testis, (13) Ovary, (14) Small intestine, (15) Colon, (16) Peripheral blood leukocyte. Multiple-tissue membranes were purchased from Clontech, Inc. (CA, USA) and hybridized with PSGR probe as suggested by the manufacturer. Bottom panel shows equal amounts of RNA were loaded in all lanes using GAPDH as a probe

interaction of the two proteins in yeast cells, we examined the activation of the *lacZ* reporter gene under the control of different GAL-4-responsive UAS and promoter element. Only yeast cells cotransformed with $G\alpha_{12}$ and the prostate-specific receptor as well as the positive control (cells transformed with p53 and large T-antigen) became blue in the β-gal assay (Figure 2c), indicating the prostate-specific receptor interacts with $G\alpha_{12}$ protein in the yeast cells.

To understand the expression patterns of the putative G-protein coupled receptor, we examined the tissue distribution of the protein using multiple-tissue

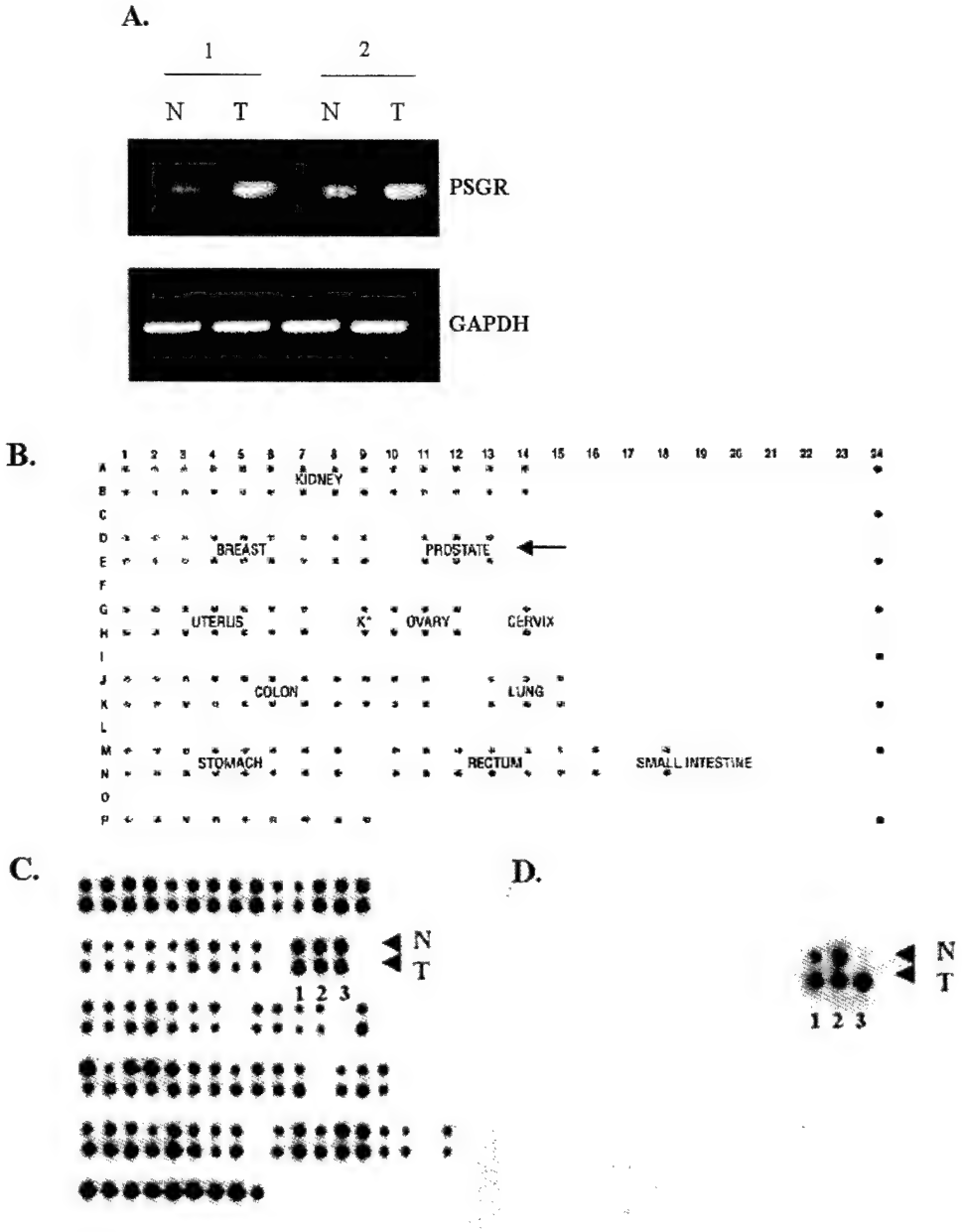

Figure 4 Over-expression of PSGR in human prostate tumor samples. (**a**) Matched cDNA pairs from human prostate normal (N) and tumor (T) tissues were obtained from Clontech. PCR were performed using primers derived from PSGR. Expression of PSGR is increased in two of the tumor samples. (**b–d**) Analysis of PSGR expression in the matched human normal/tumor expression array (Clontech Laboratory, CA, USA). (**b**) Matched normal/tumor samples were obtained from different human tissues, including kidney, breast, prostate, uterus, ovary, cervix, colon, lung, stomach, rectum, small intestine and different cancer cell lines (P) (HeLa, Daudi, K562, HL60, G361, A549, Molt-4, SW480, and Raji). (**c**) The membrane was blotted using ^{32}P-labeled Ubiquitin as a probe, demonstrating equal amount of cDNAs between the normal/tumor (N/T) samples were loaded to the membrane. (**d**) PSGR is only expressed in the prostate and is overexpressed in prostate cancer samples. The membrane was blotted using a specific PSGR probe. Three matched pairs of prostate normal tissues and adenocarcinoma tumor tissues were isolated from a 65-year-old (1), a 67-year-old (2), and a 68-year-old (3) Caucasian patient, respectively (arrow). Overexpression of PSGR was observed in two of the three pairs of prostate tumors tissues (N for normal prostate tissues; T for prostate tumor tissues)

Northern blot analysis. As shown in Figure 3, the expression of PSGR is exclusively restricted to human prostate tissues. Little or no signal was detected in other tissues (Figure 3). Expression analysis with different human tumor tissues further confirmed that the expression of PSGR is exclusively restricted to human prostate tissues (Figure 4). Therefore, PSGR is a prostate-specific G-protein coupled receptor.

To examine the potential role of PSGR in prostate cancer progression and development, we further examined the expression of the receptor in prostate tumors using PCR and Southern blot analysis with Matched Tumor/Normal prostate tissues from individual patients (Clontech Laboratories, CA, USA). As shown in Figure 4a, the expression level of the prostate-specific receptor is increased significantly in human prostate tumors as compared to that in normal prostate tissues using matched cDNA pairs from tumor and normal tissues in our PCR assays. Southern blot analysis on cDNA pairs derived from different tumor and corresponding normal tissues of individual patients further demonstrated that PSGR only expressed in human prostate tumor and normal tissues, not in other tumors or normal tissues (Figure 4b–d). The expression level of PSGR was dramatically increased in two of the three pairs of human prostate cancer samples (Figure 4d, bottom), suggesting a potential role of the receptor in human prostate tumor progression and development. Together, these experiments demonstrate that this new receptor is a prostate-specific G-protein coupled receptor (PSGR) with tumor specific overexpression in prostate cancer samples.

During the preparation of this study for publication, a paper describing the prostate-specific G-protein coupled receptor (PSGR) was independent reported by another group (Xu *et al.*, 2000). This study confirms our finding that PSGR is primarily expressed in prostate tissues and in the epithelial cells of the prostate gland. Furthermore, the expression of PSGR was dramatically increased in more than 60% of the prostate tumors, consistent with our finding that the receptor protein is overexpressed in prostate cancers.

Besides the traditional G-protein coupled signaling pathways, G-protein coupled receptors have also been shown to participate in the MAP kinase signaling pathways and the Rho/Cdc42/Rac-PAK signaling pathways in different cells and tissues (Lopez-Ilasaca *et al.*, 1997; Gutkind, 1998a; Marinissen *et al.*, 1999; Whitehead *et al.*, 2001), suggesting a direct role of G-protein coupled receptors in cell growth and proliferation processes. A number of studies demonstrated that many GPCRs and their signaling pathways have oncogenic potential and can induce tumor formation. For example, the *mas* oncogene, a putative GPCR, can induce the formation of tumors in mice (Young *et al.*, 1986). Other G-protein coupled receptors, such as serotonin 1C, muscarinic m1, m3, and m5, and adrenergic α1 receptors, have been demonstrated to transform contact-inhibited cultures of rodent fibroblasts when persistently activated (Julius *et al.*, 1989; Gutkind *et al.*, 1991; Allen *et al.*, 1991), suggesting that G-protein coupled receptors can behave as agonist-dependent oncogenes.

Studies on constitutive activation mutants of G-protein coupled receptors and G-proteins in cancers and other disease states further demonstrated that GPCRs and G-proteins play an important role in both normal and aberrant growth control (Crespo *et al.*, 1994; Radhika and Dhanasekaran, 2001; Gutkind, 1998b). Constitutive activation mutations of GPCRs have been implicated in a number of human neoplasias, including thyroid adenomas, small cell lung carcinoma, colon adenomas and carcinomas, and gastric hyperplasia and cancers (see review, Gutkind, 1998b). Furthermore, constitutively active mutants of G-protein alpha subunits have been found as transforming oncogenes, such as the Gαs as the *gsp* oncogene, Gαi as the *gip2* oncogene, and Gα_{12} as the *gep* oncogene (Landis *et al.*, 1989; Lyons *et al.*, 1990; Xu *et al.*, 1993, 1994; Chan *et al.*, 1993). Therefore, active G-protein coupled receptors and active G-protein mutants have been found in a number of human tumors and are potentially involved in the proliferation pathways of the cells (Dhanasekaran *et al.*, 1995; Radhika and Dhanasekaran, 2001). We are in the process of generating and testing the potential role of the constitutively-active PSGR receptor and its overexpression in prostate cancer.

In summary, we have identified a putative G-protein coupled receptor that interacts with Gα_{12} in yeast-two hybrid assays. The receptor is expressed exclusively in human prostate tissues. Furthermore, overexpression of the prostate-specific receptor in prostate cancers suggests this tissue-specific GPCR and its signaling pathway(s) may play an important role in human prostate tumor development.

Acknowledgments

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BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed on Form Page 2.
Photocopy this page or follow this format for each person.

NAME	POSITION TITLE
Mingyao Liu	Assistant Professor Medical Biochemistry and Genetics

EDUCATION/TRAINING (Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)

INSTITUTION AND LOCATION	DEGREE (if applicable)	YEAR(s)	FIELD OF STUDY
Hunan Normal University, Changsha, China	B.S.	1985	Biology
University of Maryland, College Park, MD	Ph.D.	1992	Cell Biology
Johns Hopkins University Medical School, MD	Postdoc	1993-94	Neuroscience
California Institute of Technology (Caltech), CA	Postdoc	1994-98	Biochemistry

RESEARCH AND PROFESSIONAL EXPERIENCE: Concluding with present position, list, in chronological order, previous employment, experience, and honors. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past three years and to representative earlier publications pertinent to this application. If the list of publications in the last three years exceeds two pages, select the most pertinent publications. DO NOT EXCEED TWO PAGES.

Professional Positions:

1993 - 1994	Postdoctoral Fellow and Research Associate, Howard Hughes Medical Institute, Johns Hopkins University, School of Medicine, Baltimore, Maryland
1994 - 1997	Research Fellow, Division of Biology, California Institute of Technology (Caltech), Pasadena, California
1997 - 1998	Senior Research Fellow, Division of Biology, California Institute of Technology (Caltech), Pasadena, California
1999-present	Assistant Professor, Center for Cancer Biology and Nutrition, Albert B. Alkek Institute of Biosciences and Technology, Texas A&M University System Health Science Center, Houston, Texas
1999-present	Assistant Professor, Department of Medical Biochemistry and Genetics, College of Medicine, Texas A&M University, College Station, Texas
1999-present	Member of the Graduate Faculty, Graduate School of Biomedical Sciences (GSBS), The University of Texas-Houston Health Science Center and The University of Texas MD Anderson Cancer Center, Houston, Texas

Awards:

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1993	HHMI Postdoctoral Fellowship, Johns Hopkins University School of Medicine, MD
2000	Scientist Development Award, American Heart Association-National
2001	Basil O'Connor Starter Scholar Award, March of Dimes Foundation

Relevant Publications (from a total of 40):

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A Rac/Cdc42 specific exchange factor, GEFT, induces cell proliferation, transformation, and migration

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Abstract

The Rho family of small GTPases, including Rho, Rac, and Cdc42, play essential roles in diverse cellular functions. The ability of Rho family GTPases to participate in signaling events is determined by the ratio of inactive (GDP-bound) and active (GTP-bound) forms in the cell. The activation of Rho family proteins requires the exchange of bound GDP for GTP, a process catalyzed by the Dbl family of guanine nucleotide exchange factors (GEFs). The GEFs have high affinity for the guanine nucleotide free state of the GTPases and are thought to promote GDP release by stabilizing an intermediate transition state. In this study, we have identified and characterized a new Rac/Cdc42-specific Dbl family guanine nucleotide exchange factor, named GEFT. GEFT is highly expressed in the excitable tissues, including brain, heart, and muscle. Low or very little expression was detected in other non-excitable tissues. GEFT has specific exchange activity for Rac and Cdc42 in our *in vitro* GTPase exchange assays and GST-PAK pull-down assays with GTP-bound Rac1 and Cdc42. Overexpression of GEFT leads to changes in cell morphology and actin cytoskeleton re-organization, including the formation of membrane microspikes, filopodia, and lamilliopodia. Furthermore, expression of GEFT in NIH3T3 cells promotes foci formation, cell proliferation, and cell migration, possibly through the activation of transcriptional factors involved in cell growth and proliferation. Together, our data suggest that GEFT is a Rac/Cdc42-specific GEF protein that regulates cell morphology, cell proliferation, and transformation.

INTRODUCTION

The Rho-related GTP-binding proteins of the Ras superfamily function as molecular switches in a variety of cellular signaling pathways and regulate diverse cellular functions, including control of cell morphology, cell migration, cell growth and proliferation, actin dynamics, transcriptional activation, apoptosis signaling, and neurite outgrowth (1-7). Among the 18 known mammalian members of the Ras superfamily, Rho A, Rac1, and Cdc42 are the most studied and well characterized ones. Each of the three members has a distinct function in cell actin cytoskeleton organization and responses (6). For example, Rho has been shown to regulate the formation of actin stress fibers and focal adhesion in fibroblasts (6,8). In contrast, Rac1 specifically induces membrane ruffling and lamellipodia formation (8,9), and Cdc42 mediates the formation of filopodia and actin microspikes (9,10). Besides the roles in actin cytoskeleton reorganization, Rho, Rac, and Cdc42 seem to be involved in a number of other cellular functions, including gene expression and transcriptional regulation (11-15), cell growth and cell cycle progression (16-21), the Jun amino-terminal kinase (JNK) signaling pathway (22,23), as well as axon guidance and extension (7,24-26).

Similar to all members of the Ras superfamily proteins, the GTP-binding/GTP hydrolysis cycle of Rho-family proteins is tightly controlled. The ability of Rho family GTPases to participate in signaling events is determined by the ratio of GTP/GDP-bound forms in the cell. Like all GTPases, they exist in an inactive (GDP-bound) and an active (GTP-bound) conformation. The activation of Rho family proteins requires the exchange of bound GDP for GTP, a process catalyzed by the Dbl family of guanine nucleotide exchange factors (GEFs) and other specific GEFs (27-29). Like the ligand-activated

seven transmembrane receptors in activation of heterotrimeric G-proteins, the GEFs have high affinity for the guanine nucleotide free state of the GTPases and are thought to promote GDP release by stabilizing an intermediate transition state (28,30).

The Dbl-family of oncoproteins is Rho-specific GEFs and contains approximately 60 distinct mammalian members (27,28). All Dbl family proteins consist of Dbl homology (DH) domain (~200 amino acid residues) and a pleckstrin homology (PH) domain (~100 amino acid residues) immediately C-terminal to the DH domain (27,31,32). DH domains interact directly with Rho GTPases to catalyze guanine nucleotide exchange by preferentially binding to Rho GTPases depleted of nucleotide and Mg^{2+} (33-38). Recent studies have determined the structure of the DH and PH domains of Tiam1 bound to nucleotide-free Rac1 and the potential mechanism to stimulate guanine nucleotide exchange of Rho GTPases by Dbl-family GEFs (30,36,39). PH domains have been found invariably followed the DH domain in the Dbl family of proteins. PH domain contains ~ 100 amino acids and has been found in a number of proteins (40,41). Although DH-associated PH domains promote the translocation of Dbl-related proteins to plasma membranes (42,43), PH domains have been shown to participate directly in GTPase binding and regulation of GEF activity in the presence or absence of phosphoinositides (28,34,44,45).

Two of the well-characterized effectors of Rac/Cdc42 GTPases are the PAK family of serine/threonine kinases and the WASP proteins. In response to physiological stimuli, the active GTP-bound Rac and Cdc42 interact with the p21 (Rac/Cdc42)-binding domain (PBD) of PAK, resulting in PAK autophosphorylation and increased kinase activity, and down stream activation of a variety of cellular functions (22,46-50).

Activation of the WASP protein by GTP-bound Cdc42 leads to the signaling cascades mediated by the WASP protein, resulting in the polymerization of cytoskeletal actin filaments (51,52). Therefore, interaction between the Rac/Cdc42 and their effectors are reversible and are dependent on the GTP/GDP binding states of the Rac and Cdc42 GTPases.

In this study, we have identified a guanine nucleotide exchange factor in both human and mouse, named GEFT, a member of the Dbl family proteins. GEFT is highly expressed in the excitable tissues, such as brain, heart, and muscle. The protein exhibited potent guanine nucleotide exchange activity on Rac1 and Cdc42, whereas little activity was observed on RhoA. Overexpression of GEFT in NIH 3T3 cells caused transformed phenotypes similar to the activation of Rac1 and Cdc42 by Vav. Furthermore, expression of GEFT induces the formation of lamellipodia, actin microspikes, and filopodia, similar to the activation of Rac and Cdc42 proteins. In addition, GEFT also stimulates the transcriptional activities of SRE, Elk1, and the c-Jun transcription factors. Taken together, our data suggest that GEFT is a specific activator preferentially for Rac1 and Cdc42 GTPases and may play important roles in cell morphology, growth and proliferation.

MATERIALS AND METHODS

DNA Constructs

The mouse GEFT fragment was initially identified by Enhanced Retroviral Mutagen (ERM) strategy (53). Briefly, we constructed Enhanced Retroviral Mutagen (ERM) vectors that contained several engineered sequences (e.g., an ERM Tag and a splice donor) controlled by a tetracycline-responsive promoter. The ERM vectors were introduced into the NIH3T3 cells. Endogenous genes can thus be randomly activated and tagged in a conditional system. NIH3T3 cells were used to screen for focus-forming genes using the ERM strategy. Full-length cDNAs encoding human *GEFTs* were obtained by screening human brain library (Clontech, CA). For mammalian expression, cDNAs encoding *GEFT* were inserted into the HindIII and SalI sites of pCMV-Tag2B (Stratagene), resulting in the plasmid of pCMV-GEFT. For expression and purification of recombinant GEFT in bacteria, GEFT was subcloned in frame into pQE-31 (Qiagen), generating His-tagged pQE-GEFT. The wild-type full-lengths of the Rho family GTPases: Cdc42, Rac1, and RhoA were subcloned into the BamH I and Sal I sites of pGEX-4T-1, a GST gene fusion vector (Amersham Pharmacia Biotech), respectively, to produce three GST-fused pGEX vectors.

Expression and purification of recombinant GEFT and GTPases

Bacterially expressed 6X His-tagged GEFT protein and GST-fusion GTPases were purified according to standard procedure of the manufacturers. *E. Coli* strain BL21 was transformed by pQE-*GEFT* and pGEX vectors respectively, grown to midlog phase at 37°C, and then induced with 1 mM IPTG for 3~4 hours. For 6X His-tagged GEFT, the

protein was purified by Ni-NTA agarose (Qiagen). For GST-fusion GTPases, the proteins were purified by GSH-agarose (Sigma). The GST-fusion proteins were in the beads or eluted in the solution containing 50 mM Tris (pH 8.0), 10 mM reduced glutathione (Sigma). All the proteins used in the assays were visualized by Coomassie blue staining after SDS polyacrylamide gel electrophoresis. The content of each protein was at least 90% pure.

Guanine Nucleotide Exchange Assays

The effects of GEFT on the dissociation of [^{3}H] GDP from the Rho family GTPases were assayed as described previously (37,54,55). Briefly, each of 1 μM eluted GTPases was incubated with 1 μM [^{3}H] GDP at 25°C in the buffer B containing 50 mM HEPES, pH7.6, 100 mM NaCl and 1 mM DTT in the presence or absence of purified 1.5 μM GEFT. To stabilize the [^{3}H] GDP–bound GTPases, the reaction mixtures were supplemented with 20 mM $MgCl_2$. After a binding equilibrium was reached (~60 min), the GDP/GTP exchange reactions were initiated by the addition of excess free 400 μM GTP (final concentration). At different time points, the reactions were terminated by filtration of 20 μl of the mixtures through nitrocellulose filters. And the filters were washed twice with the ice-cold buffer B. The amount of the radionucleotides remaining bound to the Rho GTPases (RhoA, Rac1, and Cdc42) were quantified by scintilation counting, and normalized as the percentage of [^{3}H]GDP bound at time 0. For each time point, the samples were assayed in triplicate.

Cell Culture, Transfection, and Transformation Assays

Hela, Cos-7, and NIH 3T3 cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% bovine calf serum. Cell transfection was performed using LipofectAMINE (Invitrogene) as previously described according to manufacturer's instructions (49). Cells were then allowed to grow 24 hours. For each assay, control vector encoding *LacZ* was used as a control.

For foci formation analyses, infected NIH 3T3 cells were maintained in growth medium for 12-14 days and assayed as previously described (56). Briefly, NIH3T3 cells were infected with GEFT virus or a vector (pMSCV2.1) control virus for two days. Cells were then washed with PBS, counted, and plated as shown in 6 well plates coated with 1ug/ml collagen. 5 ml of DMEM media with 10% FBS was added and changed every 3 days. Cells were allowed to grow for 14 days in a 37^0C incubator with a 95:5 air carbon dioxide mix. At the end of 14 days, cells were washed once with PBS and stained with crystal violet (0.5%), and the number of foci of transformed cells was then quantitated. Total number of foci in each well was counted with a light microscope and foci numbers were averaged for the three wells.

Transient Expression Reporter Gene Assays

Cos-7 cells were transfected by using LipofectAMINE (Invitrogene) as described previously (57). Analyses of the cell lysates of the transiently transfected cells were performed using enhanced chemiluminescence reagents from Promega as described previously (58). The reporter constructs for AP1-Luc and c-Jun-Luc were obtained from Stratagene. Reporter constructs for Elk1-luc and SAP1-luc were obtained from Dr. K.L

Guan at the University of Michigan. The data presented are the mean of three individual-transfected wells and the experiments are performed at least three times.

Northern Blotting analysis of GEFT expression in human tissues

To study the expression patterns of GEFT in different human tissues, a RNA filter comprising poly(A)-selected RNAs of multiple human tissues (Clontech, Inc.) was hybridized with specific ^{32}P-labeled cDNAs as described previously (49,58). In brief, human GEFT probe were radiolabeled with [α-^{32}P]CTP by nick translation using random primers. Probes (~ 4 x 10^7 cpm/μg) were hybridized with the RNA filter and analyzed according manufactory's protocol.

Immunoprecipitation, immunoblotting, immunocytochemistry, and fluorescence imaging

Immunoprecipitation of individual proteins was carried out as previously described (49). In brief, cell lysates (1 mg protein) were incubated with antibodies (1–10 μg) at 4°C for 1 h in a final volume of 1 ml modified RIPA buffer (10 mM sodium phosphate pH 7, 1% Triton X-100, 0.1% SDS, 2 mM EDTA, 150 mM NaCl, 50 mM NaF, 0.1 mM sodium vanadate, 4 μg / ml leupeptin, 1 mM PMSF) with constant rocking. After the addition of protein A-agarose beads, reactions were incubated at 4°C for 1 h. Immune complexes were resolved by SDS-PAGE and subjected to immunoblotting for interacting proteins.

For fluorescence labeling of the cellular components and observing cell morphology changes, 24 h after transfection, cells were plated on 10 μg/ml fibronectin-

coated glass coverslips. Then, cells were fixed with 4% paraformaldehyde for 20 min, blocked with 10% BSA, and incubated with monoclonal antibody against Flag (M2 monoclonal, Sigma). Actin filaments were labeled by rhodamine-conjugated phalloidin (Molecular Probe). Double-label immunostaining was done with appropriate fluorochrome-conjugated secondary antibodies. Fluorescent images of cells were captured on a CCD camera mounted on Olympus inverted research microscope using Ultraview imaging software (Olympus, Inc.).

Binding of GEFT to Rho GTPases and GST-PBD pull-down Assays

To determine GEFT binding affinity to the Rho GTPases, 20 μg of His-tagged protein was incubated at 4°C overnight with 20 μl of GSH-agarose beads loaded with 20 μg of each GTPase, Cdc42, Rac1 or RhoA in the absence of guanine nucleotides. The beads were washed three times with PBS. The bound proteins were separated by SDS-PAGE, and His-tagged GEFT proteins was detected by Western blotting using an anti-6X His monoclonal antibody (Santa Cruz Biotechnology).

GTPase activation assays in the cells were performed by GST-PBD pull-down assays as described previously (59-61). Briefly, cells transfected with GEFT or a control plasmid (pCMV-LacZ) were washed and lysed on the dish in 50 mM Tris-pH 7.5, 500 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, 10% glycerol, 10 MgCl2, 10ug/ml leupeptin and aprotinin, and 1 mM PMSF. GTP-bound Rac1 or Cdc42 was pulled down using the GST-PBD of PAK1 immobilized on glutathione beads. The amount of active Rac1 and Cdc42 (GTP-bound form) was detected by Western blot using specific antibodies against Rac1 and Cdc42, respectively.

Cell Proliferation Assay

Proliferation studies were carried out using the CellTiter96 AQueous One solution cell proliferation assay (Promega). Briefly, cells were transfected with GEFT or a control plasmid. Cells were plated at 500 cells/well and allowed to adhere to the plate. At indicated time points, the AQueous One solution was added to the samples and measured at 490nm.

Boyden chamber Cell Migration Assays

Cell migration/motility assays were examined using a modified Boyden chambers, as described previously (62,63). Briefly, NIH3T3 cells were stably transfected with GEFT or vector (pCMV-tag2B). The outside of filters were coated with 1 μg/ml collagen for 1 h and then washed three times with PBS. Filters were then incubated with DMEM with BSA for 1 h. Filters were then put into DMEM medium without FBS and with 0.5 ng of mouse bFGF. NIH3T3 cells expressing the receptor or vector were seeded at 20,000/well on top of the filter. Plates were incubated for 6 h. Excess cells that did not migrate through the filter were removed from the insides of the filters. Cells were then fixed with 4% paraformaldehyde for 20 min, washed three times with PBS, and then stained with crystal violet. Stained cells were examined under the microscope.

RESULTS

Identification, expression, and domain structures of GEFT

To identify genes responsible to tumorigenesis, an Enhanced Retroviral Mutagen (ERM) strategy was used to screen for foci-forming genes in NIH3T3 cells (53). One of the novel genes, mutagenized by the ERM, has shown strong oncogenic activity and was identified by RT-PCR and direct sequencing. The gene product shows sequence homology to Dbl family of guanine nucleotide exchange factors (GEFs), and was named GEFT. Subsequently, we cloned the human and mouse GEFT full-length ORF by RT-PCR and by 5'- and 3'-RACE. The mouse GEFT (*mGEFT*) sequence is 90% identical to the Human GEFT (*hGEFT*) (Fig. 1A). In contrast to hGEFT, mGEFT possesses an extra N-terminal domain. Like other family members of the Dbl proteins, GEFT has an N-terminal Rho exchange factor domain (Dbl homology domain, called DH domain) and followed by a PH domain (Fig. 1B). Sequence alignment of Dbl domains from hGEFT and other Dbl-containing proteins shows significant homology in this region, suggesting that GEFT is a potential exchange factor for the Rho-family (RhoA, Rac1, Cdc42) of GTPases (Fig. 1C). A database search found that GEFT shows 35% sequence identity with human Huntingtin-associated protein-interacting protein (Duo protein) or the spectrin-like Kalirin (64,65). GEFT also shares 35% and 60% sequence homology with the first and second DH domains of human protein Trio, respectively (66). GEFT contains 13 exons and is localized in chromasome 12q13.11, a region frequently amplified in sarcomas and brain tumors. In searching the SNP database, we found two single nucleotide polymorphisms (SNPs) in the coding region of GEFT protein: one at exon 10 with G to A substitution without an amino acid (L) change; and the other in exon

12 with nucleotide change A to G and an amino acid change Q to R (Q401R). The potential role of these SNPs in the protein is not clear at this moment.

To examine the expression of *GEFT* in human tissues, a Northern blot analysis with multiple-tissue membrane (Clonetech) was performed. As shown in Figure 2, we detected one main transcript of approximately 3 kilobase (kb), with highest expression in human brain, heart, and muscle, to less extent in small intestine, colon, liver, placenta, and lung. Weak or no expression was found in the examined immune tissues (Fig. 2). In accordance with the predicted size, a protein with a relative molecular mass (M_r) of 53,000 (53K) was identified with anti-Flag tag monoclonal antibody (M2, Sigma) in cells transfected with GEFT protein (data not shown).

Specific activation of Rac/Cdc42 by GEFT via direct interaction

To identify the Rho family of proteins that are activated by GEFT, we constructed, expressed, and purified a bacterially expressed hexahistidine-tagged human and mouse GEFT protein (DH-PH domain). Then, we analyzed the guanine nucleotide exchange activity of GEFT protein on the incorporation of cold GTP into [^{3}H]GDP-loaded RhoA, Rac1, and Cdc42, respectively (Fig. 3). As shown in figure 3, RhoA, Rac1, and Cdc42 alone did not show significant intrinsic GDP dissociation over the time period tested (8 minutes). However, addition of GEFT to the reaction stimulated rapid and complete dissociation of [^{3}H]GDP from Cdc42 and Rac1 within 2-5 min. In contrast, only 10-15% of the [^{3}H]GDP was released from RhoA after the same time period. As a control, we also examined the GEF exchange activities of Vav2 (DH domain) and Tiam-1 (DH-PH domain), members of Dbl family, on their respective exchange activities of

GTPases, Cdc42 and Rac1 (43,56,67). We found that GEFT had demonstrated similar exchange activities on Rac1 and Cdc42, as did Vav2 and Tiam-1, respectively. However, GEFT had much less exchange activity to RhoA compared to Vav2 (56). No guanine nucleotide exchange activity was found for H-Ras in our control experiment (data not shown). Taken together, our data suggest that GEFT preferentially activate the release of GDP from Rac and Cdc42 proteins, and to a much less extent to RhoA.

To further confirm that GEFT activates Rac1 and Cdc42 in the cells, we compared the amount of GTP-bound forms (active status) of Rac1 and Cdc42 in cells transfected *GEFT* or a control plasmid. To determine the level of GTP-bound Rac1 and Cdc42 in the cells, we utilized a GST-PAK1 fusion protein containing the Rac1/Cdc42 binding domain as an affinity reagent in a GST pull-down assay (59-61). PAK1 is a down-stream effector of Rac1 and Cdc42, and PAK1 binds preferentially to the active, GTP-bound forms of Rac1 and Cdc42 GTPases. As shown in figure 4A, transfection of *GEFT* in Cos-7 cells increased the Rac1-GTP level at least 5 fold compared to cells transfected with a control plasmid (pCMV-tag2B). We also found ~ 3-fold increase in Cdc42-GTP levels in cells transfected with expression plasmid encoding GEFT (Fig. 4A, right). Together, these results suggest that GEFT activate Rac1 and Cdc42 in the cell by stimulating the guanine nucleotide exchange of the two GTPases (Rac1 and Cdc42).

GEFs can be distinguished from other GTPase-interacting proteins by their ability to bind preferentially to the nucleotide-depleted state of small GTPases compared to GTP- or GDP-bound states (29,36). To test whether GEFT can directly bind to Rac1, Cdc42, and RhoA, GST-fusion protein pull-down assays were performed with GST-Rac1, GST-Cdc42, and GST-RhoA, in the absence of GTP. As shown in figure 4B,

Cdc42 and Rac1 bound His-tagged human GEFT protein in the absence of GTP, but not GST-RhoA protein. These data suggest that GEFT activate the guanine nucleotide exchange activities of Rac1 and Cdc42 through direct protein-protein interactions.

GEFT induces formation of filopodia and lamillipodia, and actin cytoskeleton rearrangement by activating Cdc42 and Rac1 GTPases in the cells

Previous studies demonstrate that in fibroblasts, Rac and Cdc42 induced the formation of lamellipodia and filopodia, respectively, while RhoA promotes stress fiber formation (3). We examine the effects of GEFT overexpression on the actin cytoskeleton reorganization in HeLa cells. As shown in figure 5, over-expression of GEFT in HeLa cells caused actin cytoskeleton rearrangement, an induction of membrane spikes and filopodia, a characteristics of Cdc42 activation by the GEFT protein (Fig. 5A-C). In addition, cells expressing GEFT displayed some membrane ruffling and formation of lamellipodia (Fig. 5D), suggesting activation of the Rac1 protein in the GEFT-transfected cells. The fact that GEFT caused the induction of filopodia, microspikes, and lamellipodia supported the possibility that overexpression of GEFT is associated with constitutive up-regulation of Cdc42 and Rac1 function.

GEFT induces foci formation and contact-independent colony growth in NIH 3T3 cells

By abrogating normal contact inhibition, some of the tumorigenic proteins have the ability to form foci of piled up transformed cells on a background monolayer of untransformed cells. To determine whether GEFT protein has the tranformation activity, we infected NIH3T3 cells with retrovirus vector encoding GEFT. As shown in figure 6,

overexpression of GEFT induces the transformed foci formation in NIH3T3 cells, while vector alone has no effect on the cells (Fig. 6A). The GEFT-induced foci contain densely packed nonrefractile cells, which is different from foci induced by Ras, similar to foci formed by active Rho family of proteins (Fig. 6B and 6C). Similar to the foci induced by another guanine nucleotide exchange factor, Vav, we observed no multi-nucleated giant cells for GEFT induced foci. The number of foci formed by GEFT in NIH3T3 cells was examined at three different cell densities. As expected, GEFT induced foci formation at all three densities (Fig. 6D), suggesting a stronger transforming ability for the GEFT protein. The transforming activity of GEFT protein, together with its ability in activation of transcription factors, suggest that this protein may play an important role in cell proliferation and tumorigenesis.

GEFT expression leads to cell proliferation and an increase in cell motility

Proliferative signaling has been associated with polypeptide growth factor receptors that possess an intrinsic protein tyrosine kinase activity as well as many G-protein coupled receptors, including thrombin, bombesin, bradykinin, substance P, endothelin, serotonin, acetylcholine, prostaglandin F2α, and lysophosphatidic acid (LPA), in a variety of cell types (reviewed by Gutkind, 1998b). The effect of GEFT on cell transformation prompted us to examine the role of GEFT in cell proliferation and in cell motility/migration. To understand the potential role of GEFT in cell proliferation and tumorigenesis, we examined cell proliferation in cells stably transfected the mouse GEFT vector using the CellTiter 96 (Promega) assay. Figure 7A shows that expression of GEFT in NIH3T3 cells significantly increased cell proliferation compared to cells

expressed the vector only, suggesting that GEFT can induce cell proliferation and tumorigenesis.

The effect of GEFT on cell morphology also prompted us to examine whether expression of this protein changes cell migration and leads to an increase in cell motility. In our experiments, we generated NIH3T3 cells stably transfected with GEFT and a control vector. When the cells were placed in a modified Boyden's chambers coated with collagen, the cells expressing GEFT migrated much faster than the ones expressing the vector alone (Fig. 7B). The number of cells migrated increased 2-3 fold in NIH3T3 cells expressing GEFT, suggesting that GEFT mediates cell motility via the activation of Rac/Cdc42 proteins.

GEFT activate Rac/Cdc42-mediated transcriptional activities

To further examine the signaling pathways activated by GEFT, we examine the ability of GEFT to stimulate Rac1- and Cdc42-mediated signaling pathways and transcription factors. It has been shown that activation of Rho family of small GTPases leads to the activation of a number of transcriptional factors in cell growth and proliferation, including the c-fos serum response element (SRE) and other transcription factors. The SRE forms a ternary complex with the transcription factor serum response factor (SRF) and ternary complex factors (TCF), such as Elk1 and SAP1. Activation of Rho-family of GTPases, RhoA, Rac1, and Cdc42, leads to transcriptional activation via SRF and act synergistically at the SRE with signals that activate TCF (12). To test whether GEFT directly affect the activation of transcription factors at the SRE, we measured the activation of GEFT on transcription factors in our cell-based transfection

assays. Cos-7 cells were transfected with luciferase reporter genes controlled by SRE, Elk1, and SAP1, together with the expression plasmids encoding Rho A, Rac1, and Cdc42. As shown in figure 7A, transfection of GEFT, together with Cdc42 and Rac1, dramatically increased SRE-luciferase activity ~100 fold and ~150 fold, respectively. GEFT also moderately activated RhoA mediated SRE activity in our transcriptional reporter assay (Fig. 7A). To further examine the effects of GEFT on TCF linked signaling pathway, we measured the activation of Ets-domain transcription factors, Elk1 and SAP1, members of the TCF complex. Similar to the activation of SRE, cotransfection of GEFT with Rac1 and Cdc42 significantly stimulated the transcriptional activities of Elk1 and SAP1 (data not shown). Whereas the stimulation of GEFT on RhoA mediated activation was significantly lower compared to Rac1 and Cdc42.

In most cell types, activation of Rac1 and Cdc42, but not RhoA, leads to the stimulation of JNK activity, and consequently, the activation of the AP-1 and c-Jun transcription factors (11,22). To investigate the effects of GEFT on the stress-regulated JNK signaling pathway, we examined the ability of GEFT to stimulate c-Jun and AP-1 transcriptional activities using a transient transcriptional reporter assays in Cos-7 cells. We transfected the control vector or vectors encoding wild type RhoA, Rac1, and Cdc42 in the presence or absence of GEFT, and then assessed the transcriptional activation of c-Jun and AP-1. As shown in figure 7B, coexpression of GEFT with Rac1 and Cdc42 significantly induced the activation of c-Jun luciferase reporter gene, whereas cotransfection of GEFT with RhoA had little stimulation of the reporter genes. Also, similar activation of AP-1 was obtained with AP1-luciferase reporter assays (data not shown). Together, these data suggest that GEFT strongly stimulates the JNK signaling

pathway and its related transcriptional factors by activating the small GTPases, Rac1 and Cdc42 in the cells.

DISCUSSION

Guanine nucleotide exchange factors (GEFs) regulate GTP-binding and regulatory proteins by converting GTPases to their biologically active state by catalyzing the exchange of bound GDP for GTP. The Dbl-family of proteins is Rho-specific GEFs that contain a Dbl domain followed by a PH domain. In our search of genes involved in tumorigenesis using Enhanced Retroviral Mutagen (ERM) strategy, we identified a new partial sequence encoding a new guanine nucleotide exchange factor and strongly promoting foci formation in NIH3T3 cells and the gene was named GEFT (53). In this research, we cloned the full-length genes encoding the human and the mouse *GEFT* and characterized the roles of this protein in Rho family of small GTPases and their signaling pathways.

We found that GEFT preferentially activated Rac1 and Cdc42 and promoted the guanine nucleotide exchange of Rac1 and Cdc42 GTPases while relatively low activity was observed with RhoA GTPase in our *in vitro* exchange assays. Moreover, GEFT activated Rac1/Cdc42-mediated transcriptional activities of SRE, Elk1, SAP1 in our transcriptional reporter gene assays. Furthermore, we demonstrated that GEFT significantly induced the activation of c-Jun and AP-1 transcription factors, downstream targets of the JNK mitogen-activated signaling pathway that is activated by Rac1 and Cdc42, but not RhoA (11,22). Therefore, GEFT may function as a specific activator for Rac1 and Cdc42 small GTPases in the cells.

The Rho family proteins have been shown to regulate actin cytoskeletal re-organization, and therefore, influencing cell shape, morphology, adhesion, cell migration

and motility (3,6). For example, RhoA promates the formation of actin stress fibers and focal adhesions, whereas Rac1 induces the formation of lamellipodia and membrane ruffling. On the other hand, Cdc42 promotes formation of microspikes on cell surface and filopodia development in the cells. In our experiments, we observed that expression of GEFT promotes cell morphology change and actin cytoskeleton reorganization. Like activation of Cdc42 and Rac1, we found that GEFT induced the formation of microspike, filopodia, and lamellipodia structure in cells overexpressing the proteins, suggesting that GEFT activate Rac1- and Cdc42-mediated signal pathways in the cells.

The fact that GEFT is highly expressed in the brain and the heart, and that GEFT is localized on Chromasome 12q13.11, a region frequently amplified in sarcomas and brain tumors, suggest that the protein may play a potential role in brain tumor and other tumors. In this study, we demonstrated that overexpression of GEFT in NIH3T3 cells strongly induced the formation of foci, similar in morphologic appearance to the ones induced by activated Rac and Cdc42 signaling pathway, suggesting the tumorigenic potential of the GEFT protein. We also demonstrated that GEFT strongly activated a number of transcription factors that mediated the expression of genes involved in cell growth, proliferation, and survival. These cellular functions are important in tumorigenesis. The exact roles of this protein in brain tumor and other tumors are under further investigation.

There is a great deal of evidence that Rho family of GTPases play an important role in neuronal morphogensis (7,11,22). During neuronal development, neuronal precursor cells migrate and then differentiate, extending axons and dendrites to specific

regions to form synapses with appropriate target cells. Several GEFs, such as Tiam1, Trio, Ephexin, and Kalirin, have been implicated in neuronal morphogenesis, growth cone guidance, and neuronal dendritic spines (68-72). We have demonstrated that GEFT is highly expressed in human brain and GEFT can induce the formation of lamillipodia, microspikes, and filopodia in the cells. The potential roles of this brain specific GEF protein in neuronal morphogenesis and differentiation, axon guidance, and dendritic spines are under investigation.

A variety of extracellular stimuli have been found to activate the Rho family of GTPases, including growth factors, cytokines, LPA, interleukins, and matrix components via receptor tyrosine kinases, G-protein coupled receptors, and integrin receptors, respectively. How different signaling pathways directly link the extracellular stimuli to the intracellular signal change and consequently, gene expression, is one of the key questions. Protein phosphorylation or membrane association and protein-protein interaction through the PH domain of GEFs has been shown to activate GEF proteins upon stimulation by extracellular stimuli (44,45,73-75). Domain and motif analysis of GEFT protein indicates that GEFT protein has a PH domain directly after the guanine nucleotide exchange domain and several potential protein phosphorylation sites that can be regulated by pathways coupled to different cellular protein kinases.

In summary, we have identified and characterized a guanine nucleotide exchange factor, preferentially for Rac1 and Cdc42, named GEFT. The protein is highly expressed in human brain and heart, very weakly in other tissues. Overexpression of GEFT in the cell induces cell morphology change, cytoskeleton reorganization, and the formation of

microspikes, filopodia, and lamilliopodia, characteristics of Rac1/Cdc42 activation. Furthermore, overexpression of GEFT in NIH3T3 cells promotes the induction of foci formation and tumorigenesis, possibly by activating Rac1 and Cdc42 signaling pathways and transcription factors in the cells. The mechanism of GEFT activation and its roles in neuronal cells and tumors need to be established in future studies.

ABBREVIATIONS

The abbreviations used are: GEFs, Guanine nucleotide exchange factors; GAPs, GTPase activating proteins; PH, Pleckstrin homology; PCR, polymerase chain reaction; GST, glutathione *S*-transferase; Dbl, Diffuse B-cell lymphoma; DH, Dbl homology.

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Figure Legends

Figure 1. GEFT domain structure and comparison of Dbl homology (DH) domains. **A**. Sequence comparison of human and mouse GEFT proteins. Identical amino acids are marked *. GenBank accession numbers are AF487514 for human and AF487515 for mouse. **B**. Domain structure of human GEFT, including the N-terminal DH domain (Rho GEF domain, also called DH domain) followed by the PH (pleckstrin homology domain). Mouse GEFT has similar domain structure and an extra N-terminal region (not shown). **C**. Multiple sequence alignment of DH domains from GEFT and other member of the Dbl family members, Tiam1, Kalirin (Duo), and UNC-73 (64,65,67,76). Bold letters indicate identical amino acids.

Figure 2. Expression of GEFT in human tissues. Northern blot analysis of *GEFT* expression in multiple human tissues using multiple tissue northern blot membrane containing premade poly A + RNA (Clontech Inc.). The membrane was hybridized with ^{32}P-labeled GEFT and β-actin probes, respectively. A single band at approximately 3 kilobases (kb) was detected.

Figure 3. GEFT preferentially activates the guanine nucleotide exchange activities of Rac1 and Cdc42. Stimulation of GDP dissociation from RhoA, Rac1, and Cdc42 by GEFT was performed using purified bacterial expressed proteins. Time-dependent study for the dissociation of [^{3}H]GDP from purified recombinant GST-RhoA (**A**), GST-Rac1 (**B**), and GST-Cdc42 (**C**) in the presence or absence of His-tagged GEFT. Experiments were performed in triplicates. Shown are representative of three independent assays.

Figure 4. GEFT activates and binds to Rac1 and Cdc42. **A**. Activation of Rac1 and Cdc42 by increasing the levels of GTP-bound forms of Rac1 and Cdc42, respectively, in cells expressing GEFT. The amounts of activated Rac1-GTP and Cdc42-GTP were determined by GST-pull down assays using GST-PAK1 domain (59-61). Cells transfected with GEFT (+) and a control plasmid (pCMV-*Tag2B*) (-) were lysated and the amount of GTP-bound Rac1 and Cdc42 were precipitated with GST-PAK. The proteins were separated by 12% SDS-polyacrylamide gel electrophoresis. The amounts of Rac1 and Cdc42 proteins were visualized by Western blot analysis using specific anti-Rac1 and Cdc42 antibodies, respectively. Western blot was also performed to verify equal amount of endogenous Rac1 and Cdc42 expression used in the assays (A, bottom, Rac1 and Cdc42). **B**. GEFT directly binds to Cdc42 and Rac1 *in vitro*. Bacterial expressed and purified GST-RhoA, Cdc42, and Rac1 were assayed for *in vitro* binding of His-tagged GEFT without the addition of GTP as described in materials and methods. His-tagged GEFT binds to Rac1 and Cdc42 in the pull-down assays. Bottom of Fig. 4B shows equal amounts of GST-fusion proteins (GST-RhoA, GST-Rac1, and GST-Cdc42) were used in the assays.

Figure 5. GEFT induces the formation of membrane microspikes, filopodia, and lamellipodia in the cells. **A-B**. Formation of membrane microspikes in cells transfected with Flag-tagged GEFT. **C-D**. Induction of filopodia and lamellipodia in transfected Hela cells. Immunostaining of GEFT and actin cytoskeleton in Hela cells transfected with pCMV-Flag-GEFT. Cells were plated on fibronectin-coated coverslips, then fixed

and immunostained with specific anti-Flag monoclonal antibody M2 (Sigma) for the Flag- GEFT (left of figures). Actin cytoskeleton was stained with rhodamine-phalloidin (middle of figures). Shown in the right are overlay pictures of GEFT (left) and actin cytoskeleton (middle).

Figure 6. Induction of foci formation in NIH3T3 cells by GEFT expression. NIH3T3 cells were infected with a retrovirus vector encoding GEFT or a control plasmid (pMSCV2.1, Clontech Inc., CA). **A**. Cells expressing the control plasmid did not form any foci in the assays. **B-C**. Induction of foci formation in GEFT infected cells. Photographs of foci formation were taken 10 days after NIH3T3 cells were infected with GEFT. The experiments were performed three times and shown are representatives from foci formed by the GEFT infected NIH3T3 cells. **D**. The number of foci formed in GEFT expressing cells and vector control cells. Three individual experiments with different numbers of cells were plates and the numbers of foci formed were counted for individual cell population. Shown are the mean and standard error of three independent assays.

Figure 7. GEFT promotes cell proliferation and cell migration. NIH3T3 cells infected with either control vector or GEFT were used in a cell proliferation and migration assays. **A**. Expression of GEFT induced cell proliferation. Cells were transfected with GEFT and a control vector, respectively. Cells were plated at 500 cells/well and allowed to adhere to the plate. At indicated time points, the AQueous One solution was added to the samples and measured at 490nm 2 hrs later. The experiments were repeated three times and data shown are the means of three independent repeats. **B**. Cell migration/motility

assay using a modified Boyden Chamber approach. Chemoattractant was placed into the base wells and separated from the top wells by an 8.0 μm pore Nucleopore PVP-F polycarbonate membranes (Corning Separations, MA, USA), pre-coated 1 hr with collagen (100 μg/ml) (Collaborative Biomedical, MA, USA). 2x 10^4 cells were pipetted into each upper well of the chamber, which was then incubated for 6 h at 37°C in a 5% CO_2 humidified incubator. Non-migrated cells were removed and membranes were fixed and stained. The number of cells migrating per well was counted microscopically, with three wells per condition. Then, the mean and standard deviation or standard errors were calculated.

Figure 8. Activation of transcriptional factors by GEFT in the cells. **A**. GEFT activates the serum response element (SRE) mediated by the Rho family GTPases. Rho family of proteins moderately activates the SRE-Luc reporter gene, whereas co-transfection of GEFT dramatically stimulates the Cdc42- and Rac1-mediated SRE in the reporter assays. **B**. Activation of Rac1- and Cdc42-mediated c-Jun transcriptional activities by GEFT. Cos-7 cells were transfected with individual reporter plasmid, wild type or dominant-active (DA for dominant active) RhoA, Cdc42, and Rac1. pCMV-Flag-tagged GEFT is used in the transcriptional reporter assays. Data shown are an average of three repeats. Similar experiments were performed at least three times.

A.

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                * * * ** ****************** ** ********* ****** ******* ** *
Human       2   LEPALATGEELPELTLLTTLLEGPGDKTQPPEEETLSQAPESEEEQKKKALERSMYVLSE
Mouse       147 LGPTLTTGQELPELTLLTTLLEGPGDKAQPAEEETLSQAPKNEEEQKKMALERSMFVLGE

                *****  * *************** **********************************
Human       62  LVETEKMYVDDLGQIVEGYMATMAAQGVPESLRGRDRIVFGNIQQIYEWHRDYFLQELQR
Mouse       207 LVETERTYEDDLGQIVEGYMATMATQGVPESLRGRDRIVFGNIQQIYEWHRDYFLQELQQ

                ********************************** ************************
Human       122 CLKDPDWLAQLFIKHERRLHMYVVYCQNKPKSEHVVSEFGDSYFEELRQQLGHRLQLNDL
Mouse       267 CLKDPDWLAQLFIKHERRLHMYVVYCQNKPKSEHVLSEFGDSYFEELRQQLGHRLQLNDL

                ********************** *** **  ******************* *********
Human       182 LIKPVQRIMKYQLLLKDFLKYYNRAGMDTADLEQAVEVMCFVPKRCNDMMTLGRLRGFEG
Mouse       327 LIKPVQRIMKYQLLLKDFLKYYRRAGKDTEELEQAVEVMCFVPKRCNDMMSLGRLRGFEG

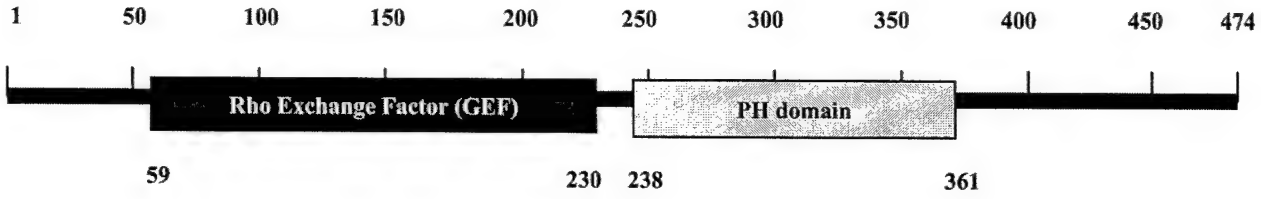
                ************** ************************************ *** *****
Human       242 KLTAQGKLLGQDTFWVTEPEAGGLLSSRGRERRVFLFEQIIIFSEALGGGVRGGTQPGYV
Mouse       387 KLTAQGKLLGQDTFLVTEPEAGGLLSSRGRERRVFLFEQIIIFSEALGGGGRGGAQPGYV

                ****************** ********************** *** ****** *******
Human       302 YKNSIKVSCLGLEGNLQGDPCRFALTSRGPEGGIQRYVLQAADPAISQAWIKHVAQILES
Mouse       447 YKNSIKVSCLGLEGNLQGNPCRFALTSRGPEGGIQRYVLQASDPAVSQAWIKQVAQILES

                *************************** ** ******   ** ** * ************
Human       362 QRDFLNALQSPIEYQRRESQTNSLGRPRGPGVGSPGRIQLGDQAQGSTHTPINGSLPSLL
Mouse       507 QRDFLNALQSPIEYQRRESQTNSLGRPGGPWVGSPGRMRPGDLAQASMHTPINGSLPSLL

                * * *** * ***** *** * ** *****   **  ****************
Human       422 LSPKGEVARALLPLDKQALGDIPQAPHDSPPVSPTPKTPPCQARLAKLDEDEL
Mouse       567 LLPRGEVSRVLLPLDTQALSDTPQTPHDSPAL-PTVNTPPCQARLAKLDEDEL
```

B.



C.

```
                                10        20        30        40        50        60
                       ....*....|....*....|....*....|....*....|....*....|....*....|
Consensus       1      VLKELLQTERNYVRDLKILVEVFLKPLKKEAK---LLSPDEVETL-FGNIEEIYEFH-RI 55
hGEFT           58     VLSELVETEKMYVDDLGQIVEGYMATMAAQGV---PESLRGRDRIvFGNIQQIYEWH-RD 113
Tiam1           15     VICELLETERTYVKDLNCLXERYLKPLQKET----FLTQDELDVL-FGNLTEXVEFQ-VE 68
Kalirin         1278   IMAELLQTEKAYVRDLHECLETYLWEMTSGVeeipPGILNKEHII-FGNIQEIYDFHnNI 1336
UNC-73          1813   VLMELVETEQDYVKDLTSVVEGYIGNLNKMDl--pADLVGKDKII-FANIVNILEFHkTN 1869

                                70        80        90        100       110       120
                       ....*....|....*....|....*....|....*....|....*....|....*....|
Consensus       56     -FLDELEKRVEEW----------DDSGDRIGDVFLKL-EELFKIYSEYCSNHPDALEL 101
hGEFT           114    yFLQELQRCLKDP----------DW----LAQLFIKH-ERRLHMYVVYCQNKPKSEHV 156
Tiam1           69     -FLKTLEDGVRLVpdleklekvdqfKKVLFSLGGSFLYY-ADRFKLYSAFCASHTKVPKV 126
Kalirin         1337   -FLKELEKYEQL---------------PEDVGHCFVTW-ADKFQMYVTYCKNKPDSNQL 1378
UNC-73          1870   -FLKEIEKCSEN---------------YEAAGAAFVKYeRRLHTLYVTYCQNKPKSDYL 1912

                                130       140       150       160       170       180
                       ....*....|....*....|....*....|....*....|....*....|....*....|
Consensus       102    LKKLKKKNKRFQKFLKEIESNPNCRRLELESLLLKPVQRLTKYPLLLKELLKHTPPDHED 161
hGEFT           157    VSEFGDS---YFEELRQQLGH----RLQLNDLLIKPVQRIMKYQLLLKDFLKYYNRAGMD 209
Tiam1           127    LVK-AKTDTAFKAFLDAQNPRQQHS-STLESYLIKPIQRVLKYPLLLRELFALTDAESEE 184
Kalirin         1379   ILE---HAGTFFDEIQQRHGLAN----SISSYLIKPVQRVTKYQLLLKELLTCCE---EG 1428
UNC-73          1913   LAQ-----DDFEAFFADTKAKLGHk-VALCDLLIKPVQRIMKYQLLLKDILKFTERAKDK 1966

                                190       200
                       ....*....|....*....|
Consensus       162    REDLKKALDAIKELASQVNE 181
hGEFT           210    TADLEQAVEVMCFVPKRCND 229
Tiam1           185    HYHLDVAIKTXNKVASHINE 204
Kalirin         1429   KGELKDGLEVMLSVPKKAND 1448
UNC-73          1967   TDTLKKALQVMHVVPKACDD 1986
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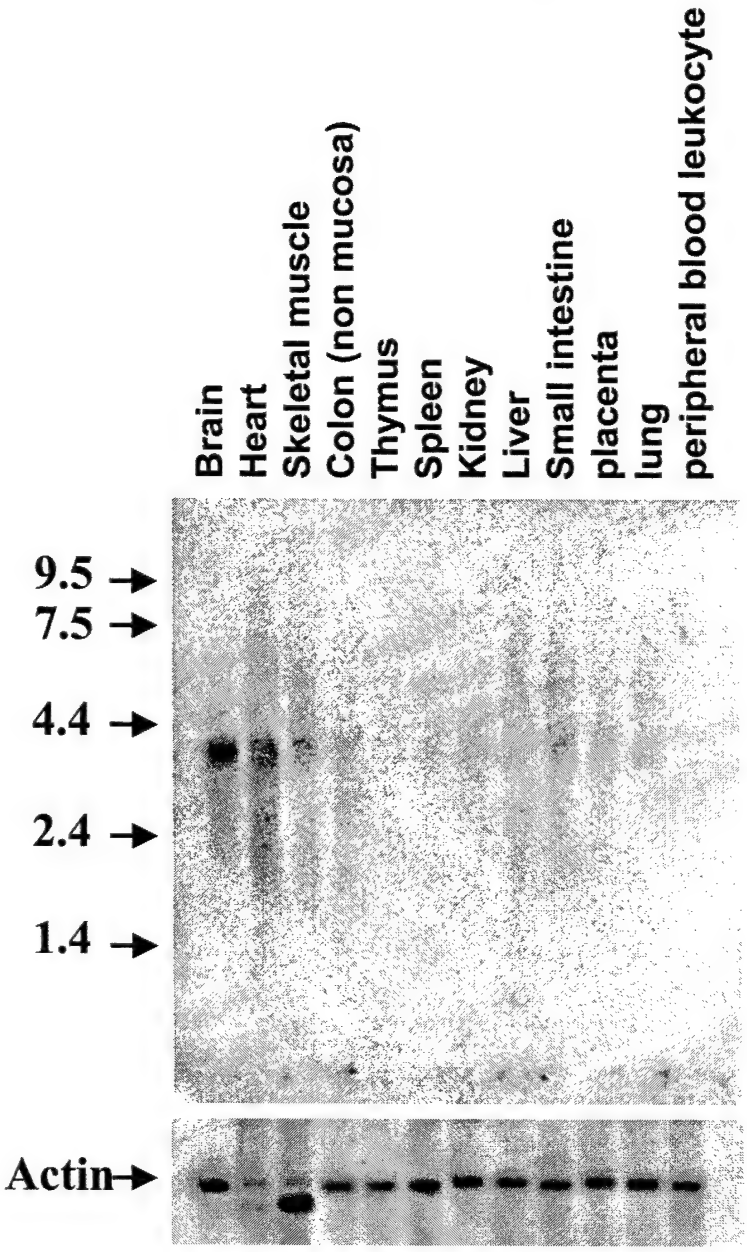
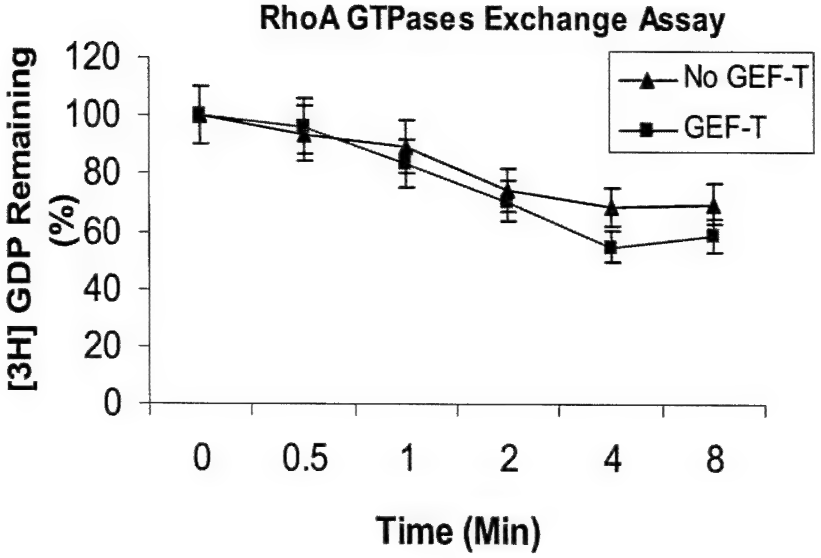

Brain
Heart
Skeletal muscle
Colon (non mucosa)
Thymus
Spleen
Kidney
Liver
Small intestine
placenta
lung
peripheral blood leukocyte
9.5
7.5
4.4
2.4
1.4
Actin

A.


RhoA GTPases Exchange Assay
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120
100
80
60
40
20
0
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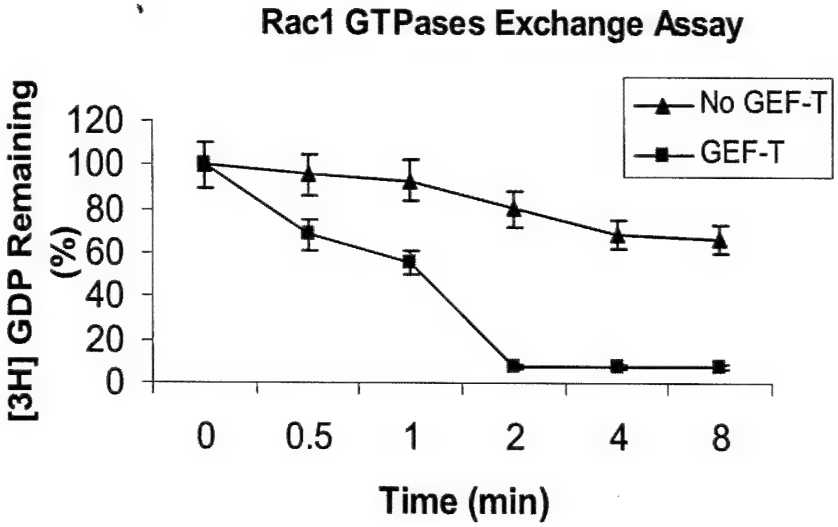
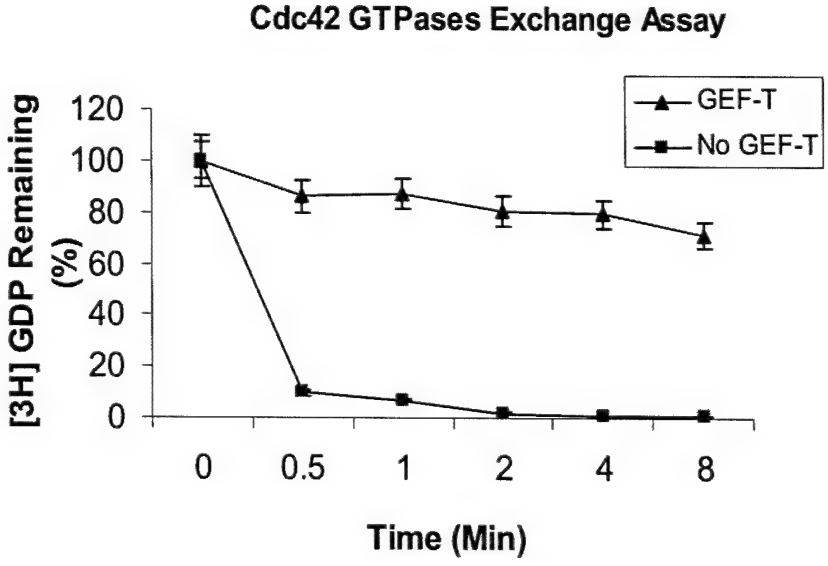
GEF-T
0
0.5
1
2
4
8
Time (Min)

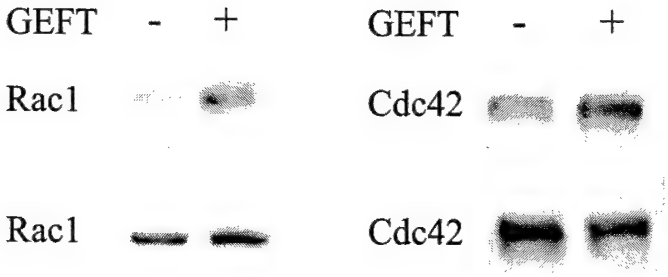
B.


Rac1 GTPases Exchange Assay
[3H] GDP Remaining (%)
120
100
80
60
40
20
0
No GEF-T
GEF-T
0
0.5
1
2
4
8
Time (min)

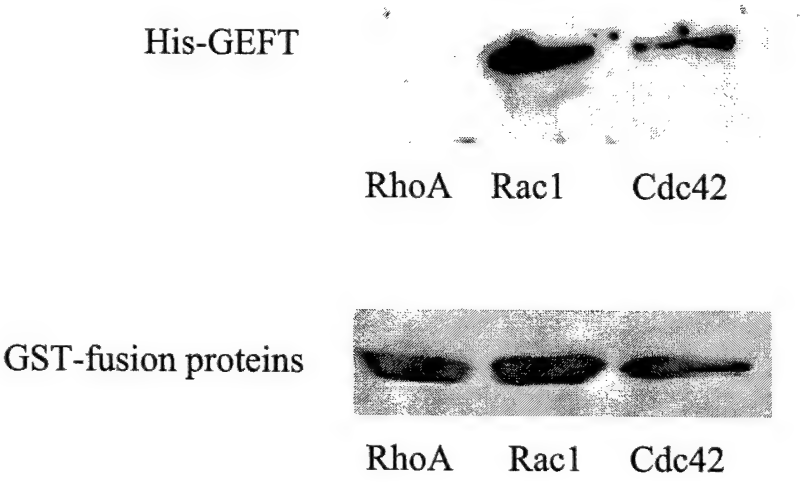
C.


Cdc42 GTPases Exchange Assay
[3H] GDP Remaining (%)
120
100
80
60
40
20
0
GEF-T
No GEF-T
0
0.5
1
2
4
8
Time (Min)

A.

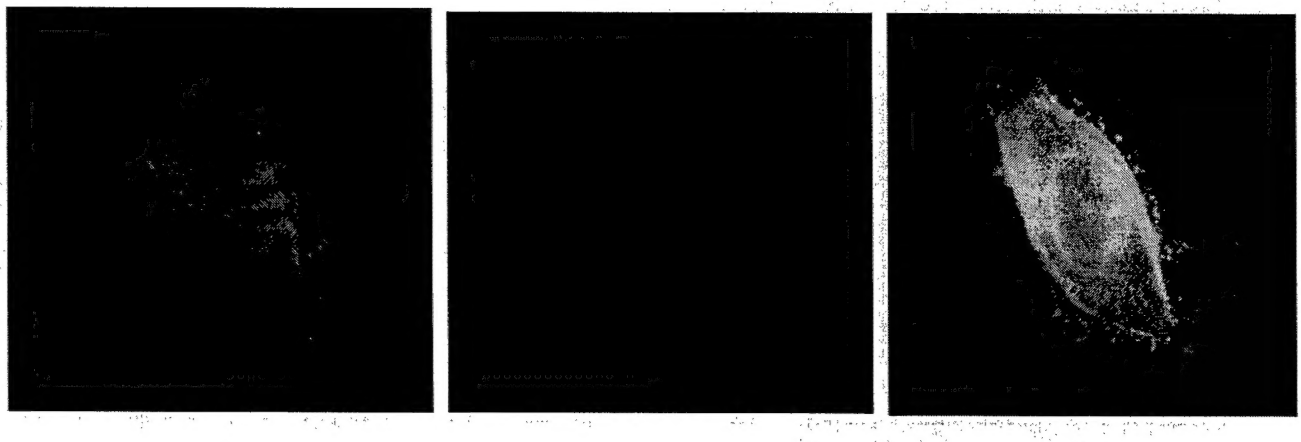

GEFT - +
Rac1
Rac1
GEFT - +
Cdc42
Cdc42

B.

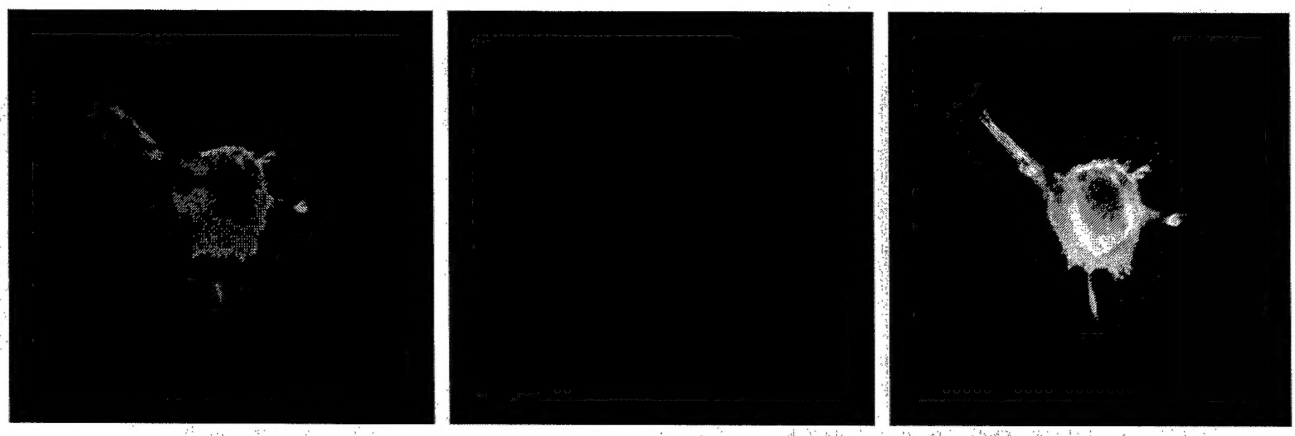

His-GEFT
RhoA Rac1 Cdc42
GST-fusion proteins
RhoA Rac1 Cdc42

A.

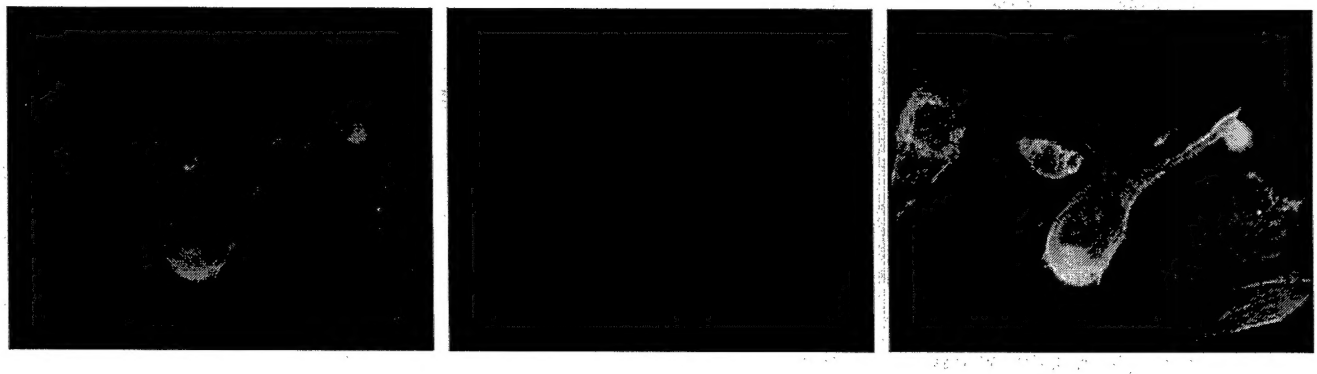
B.



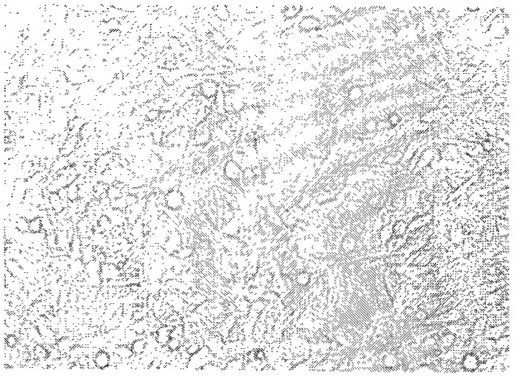
C.



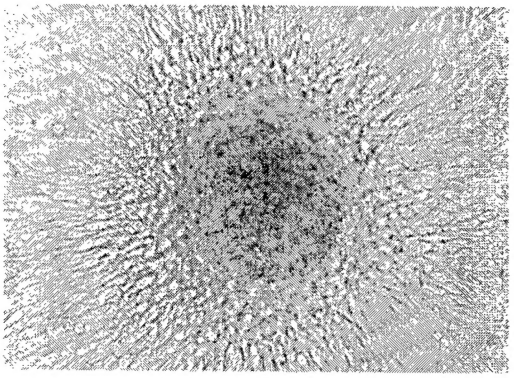
D.



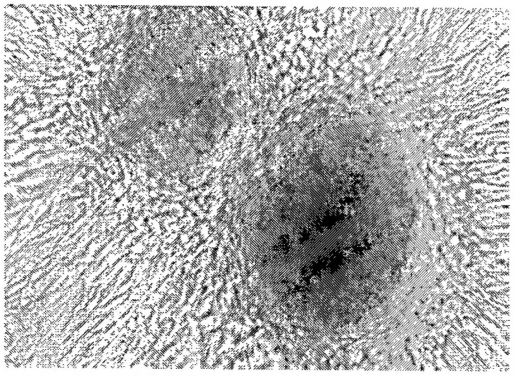
A.



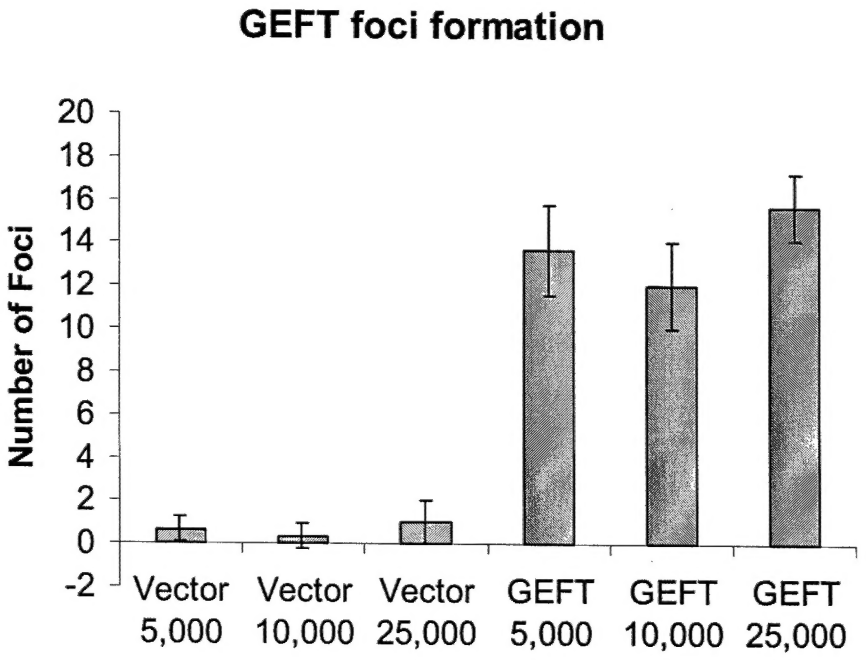
B.



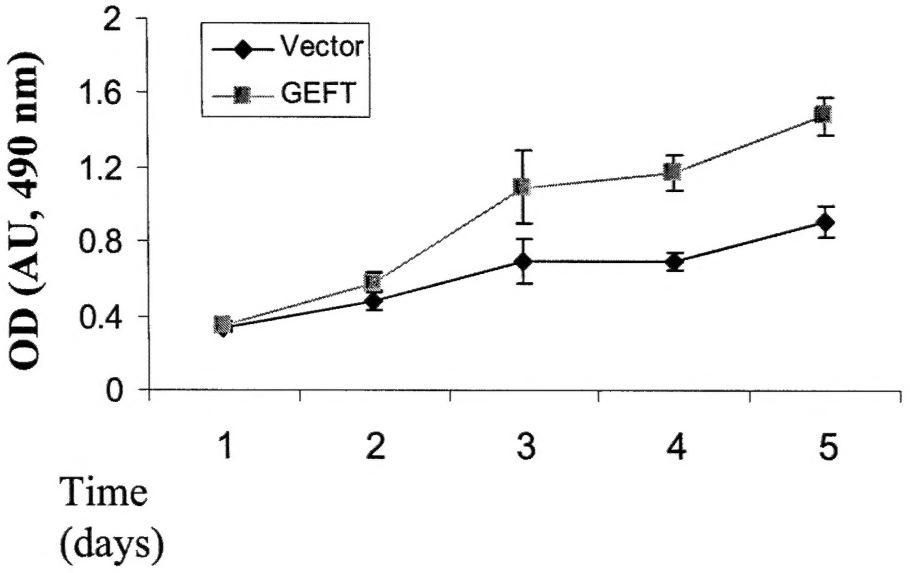
C.



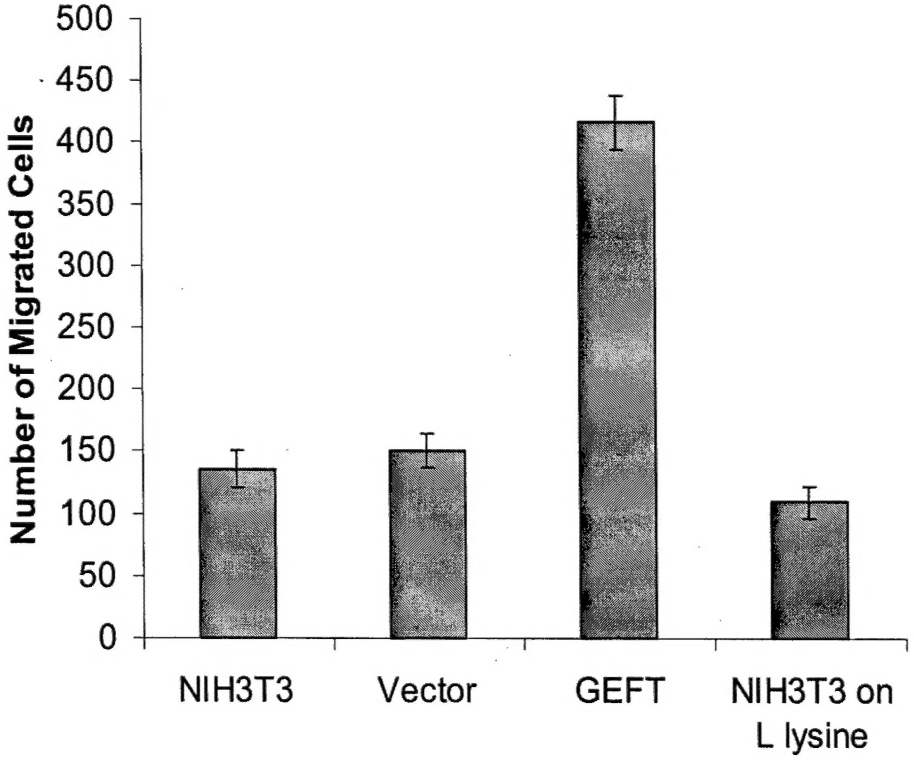
D.


GEFT foci formation
Number of Foci
20
18
16
14
12
10
8
6
4
2
0
-2
Vector 5,000
Vector 10,000
Vector 25,000
GEFT 5,000
GEFT 10,000
GEFT 25,000

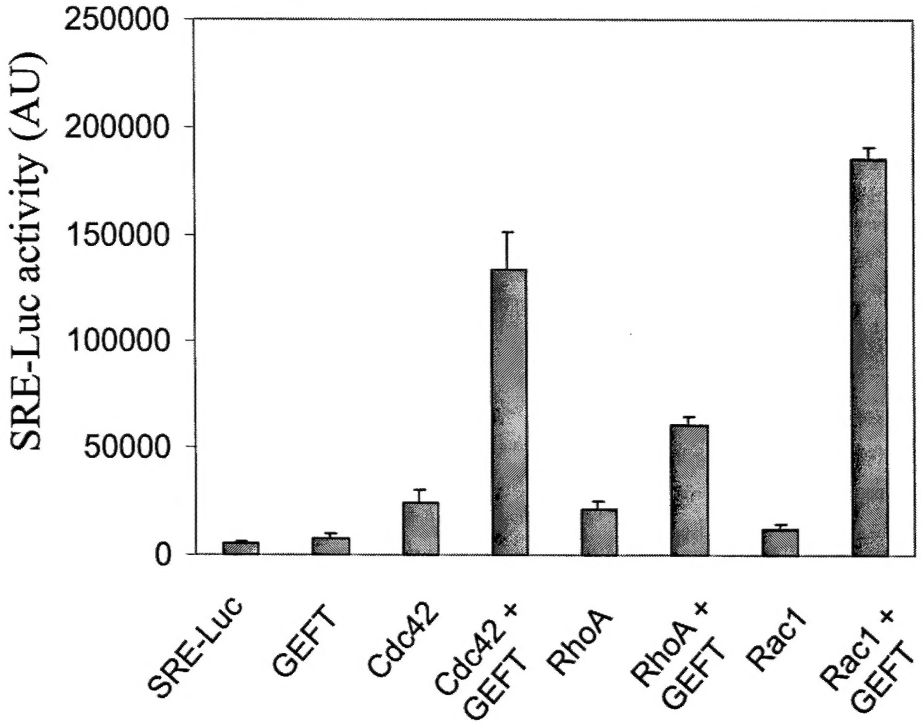
A.


OD (AU, 490 nm)
2
1.6
1.2
0.8
0.4
0
Vector
GEFT
1
2
3
4
5
Time
(days)

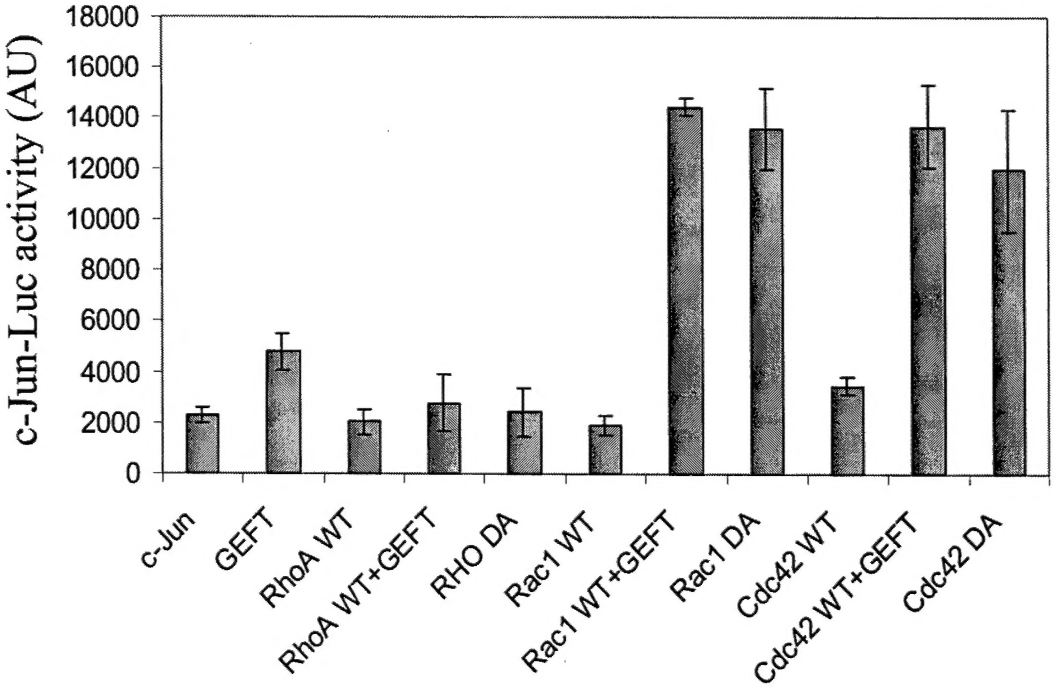
B.


Number of Migrated Cells
500
450
400
350
300
250
200
150
100
50
0
NIH3T3
Vector
GEFT
NIH3T3 on
L lysine

A.


SRE-Luc activity (AU)
250000
200000
150000
100000
50000
0
SRE-Luc
GEFT
Cdc42
Cdc42 + GEFT
RhoA
RhoA + GEFT
Rac1
Rac1 + GEFT

B.


c-Jun-Luc activity (AU)
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
c-Jun
GEFT
RhoA WT
RhoA WT+GEFT
RHO DA
Rac1 WT
Rac1 WT+GEFT
Rac1 DA
Cdc42 WT
Cdc42 WT+GEFT
Cdc42 DA